Spinal sensory processing in the human infant:

Development of the flexion withdrawal reflex

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Thesis submitted for the degree of Doctor of Philosophy

University College London

2011

Declaration

The work in this thesis was conducted in the Department of Neuroscience, Physiology and Pharmacology at University College London, and in the Elizabeth Anderson and Obstetrics Wing at University College Hospital. I, Laura Louise Cornelissen, confirm that the work presented in this thesis is my own. Where other information has been derived from other sources, I confirm that this has been indicated in the thesis.

Laura Louise Cornelissen

March 2011

Abstract

Immature spinal sensory reflexes have lower mechanical thresholds and are poorly coordinated and exaggerated compared to adult reflexes. However, little quantitative data is available on how these spinal sensory circuits develop in the human infant. This thesis investigates the development of cutaneous flexion withdrawal reflexes in preterm and full-term human infants following noxious and non-noxious stimulation of the heel, and tests whether flexion withdrawal reflex activity is modulated by the commonly administered analgesic, oral sucrose, in a randomised controlled trial.

The studies were undertaken in infants aged 28-45 weeks gestation (GA), in-patients at University College Hospital, London. The noxious stimulus was a clinically required heel lance; non-noxious stimulation was either a light touch of the heel or application of calibrated von Frey hairs to the heel. Flexion withdrawal reflex activity was recorded with surface EMG electrodes placed over the biceps femoris muscle. Video recordings of facial expression were recorded for clinical pain assessment.

Noxious heel lance evoked bilateral withdrawal reflex activity across all ages, but the peak amplitude was greater in preterm infants (<37 weeks GA) than full-term infants ($\geq$37 weeks GA). Nociceptive reflex activity occurred even when there was no change in facial expression. Non-noxious touch evoked a significantly smaller reflex response than noxious stimulation.

The von Frey hair cutaneous mechanical threshold for flexion withdrawal reflex activity increased with gestational age. Repeated stimulation with von Frey hairs at suprathreshold intensities caused greater habituation in full term than preterm infants.

Flexion withdrawal reflex properties were not altered by administration of oral sucrose before a heel lance, despite significant reduction in clinical facial expression scores.

In conclusion, human spinal sensory circuits undergo significant postnatal development that alters their behavioural responses to touch and pain. The commonly used neonatal analgesic, oral sucrose, does not affect sensory processing at the level of the spinal cord.

Acknowledgements

Over the course of the last four years I have been fortunate to meet and work with many wonderful people. Completing a doctoral programme and indeed, writing up a thesis is by no means a simple undertaking, however it would be not possible without the personal and practical support of numerous people, of whom I now take pleasure in thanking.

First of all I would like to thank my PhD supervisor Maria Fitzgerald for her excellent advice, teaching and encouragement. I have been motivated by her enthusiasm and drive to further understand the development of the nervous system and pain processing throughout my PhD programme and look forward to future research in this area. Secondly, my sincere thanks to my secondary supervisor Stewart Boyd, whom I enjoyed many, many hours of discussion with, and whose gentle encouragement has massively built up my confidence as a scientist.

I wish to thank Judith Meek, for enabling the collaboration between UCL and the neonatal unit at UCH, without this it would not have been possible to conduct this work. I would like to thank all the parents and infants who participated in these studies.

Many thanks to Rebeccah Slater, who taught me valuable skills in conducting and interpreting electrophysiological recordings on the neonatal unit; along with many hours of discussion and her excellent teaching. I wish to thank Lorenzo Fabrizi, for proving invaluable MatLab code, being a mentor and good friend. Many thanks to Debbie Pattern, Jan Yoxen and Anne Canterella, the neonatal research nurses who meticulously analysed videos for PIPP analysis, and who I have had the pleasure of working alongside on the neonatal unit. Many thanks to Alan Worley, who built the event marking system that enabled the stimuli to be time-locked to the EMG and video recordings, and for his advice on numerous aspects of the recording system set-up and support along the way.

To the members of the Fitzgerald and Hunt laboratories, past and present, particularly to Gareth Hathway and Stephanie Koch who provided great advice on the electrophysiological and developmental aspects of this thesis, and to Gemma Williams for numerous discussions and proof-reading this thesis. Also to my science buddy, Katherine Quick, for the useful advice whilst writing up and crucially, for making me laugh.

Thank you to the Medical Research Council who financially supported this work.

Many thanks to my friends who have always been there: particularly to Melanie Earp, Alexis Hopwood, Laura White, Alison Twelvetrees and Dan Bose for providing a continuous supply of tea, and to my housemates, especially Adrian Barker, Jennifer Fox and Ben Todd who have ensured I have been fed and provided a continuous supply of entertainment.

I would like to thank my family for their continual support and encouragement over the years. Finally, I would like to thank Dan Ward for his thorough proof reading, along with his kind support in dealing with 'those' moments during writing up of this thesis.

Thank you all.

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Abbreviations

μV	microvolts
AD	After-stimulus Discharge
AMPA	α-Amino-3-hydroxy-5-Methyl-4-isoxazolePropionic Acid
ANOVA	Analysis Of Variance
Base	Baseline activity
Bpm	Beats Per Minute
CI	Confidence Interval
CONSORT	Consolidated Standards Of Reporting Trials
CNS	Central Nervous System
CVLM	Caudal Ventrolateral Medulla
DRG	Dorsal Root Ganglion
E	Embryonic day
EvR	Evoked Response
EEG	Electroencephalography
EMG	Electromyography
EMLA	Eutectic Mixture of Local Anaesthetic
EPSC	Excitatory Post-Synaptic Current
$F_{(x,y)}$	F distribution with *x* degrees of freedom in the numerator and *y* degrees of freedom in the denominator
g	grams
GA	Gestational Age
GABA	γ-Aminobutyric Acid
H	Hypothalamus
LED	Light-Emitting Diode
LPb	Lateral Parabrachial area
ml	millilitres
ms	milliseconds
mV	millivolts
MHRA	Medicines and Healthcare products Regulatory Agency
n/N	number of infants in each group/total Number of infants
Non-CTIMP	Non-Clinical Trial of an Investigational Medicinal Product

NICU	Neonatal Intensive Care Unit
NK1	Neurokinin 1
NMDA	N-Methyl-D-Aspartate
NTS	Nucleus Tractus Solitarius
P	Postnatal day
PAG	Periaqueductal Grey
PIPP	Premature Infant Pain Profile
QST	Quantitative Sensory Testing
RMS	Root Mean Square
RS	Repetitive Stimulation
RVM	Rostral Ventromedial Medulla
s	seconds
SCBU	Special Care Baby Unit
SD	Standard Deviation
SEM	Standard Error of the Mean
SMT	Spinomesencephalic Tract
SRT	Spinoreticular Tract
t	Time
T_c	Control Threshold
T_{rs}	Post-Repeated Stimulation Threshold
TC	Transitional Care
TFS	Total Facial Score
TRP	Transient Receptor Potential ion channel family
UCH	University College Hospital
vFh	von Frey hair

Chapter 1

General Introduction

1 Introduction

The first site in the mammalian central nervous system (CNS) for processing cutaneous sensory information is the spinal cord and equivalent trigeminal areas in the brainstem. Primary afferent fibres carry tactile and nociceptive information from the periphery to the dorsal horn of the spinal cord for integration by a complex network of spinal interneurons, projection neurons and motor neurons. Information is then transmitted to subcortical centres including the brainstem, thalamus and on to the cortex where sensory-discriminative and emotive aspects of the sensory experience are processed. In addition, local neuronal circuits in the spinal cord are involved in the coordination of reflexes. Of particular interest is the flexion withdrawal reflex, a protective nocifensive behaviour that withdraws an affected limb away from imminent danger. This reflex was first described by Sherrington and has been used extensively as an indirect measure of spinal nociceptive activity and excitability (Sherrington, 1906; Woolf *et al.*, 1984; Woolf *et al.*, 1986).

While our understanding of the cutaneous flexion reflex in adult spinal cord is relatively comprehensive, much less is known about its properties in the neonate. The neonatal central nervous system is capable of processing sensory information from before birth but undergoes further maturation throughout early life. Cutaneous reflexes in rat-pups and kittens, have lower thresholds, and larger cutaneous receptive fields, than those in the adult and result in comparatively imprecise and poorly directed avoidance behaviour, (see Fitzgerald, 2005). While some studies of the flexion reflex have been carried out in the human infant, most research in this area has relied upon visual observations of limb activity, which are subjective in nature and lack the comprehensive quantification of neurophysiological recordings (Abdulkader *et al.*, 2008b; Andrews *et al.*, 1999; Andrews *et al.*, 1994).

A better understanding of the postnatal development of cutaneous reflexes in human infants is important for two reasons. Firstly, it will improve our basic knowledge of the development of CNS processing of tactile and nociceptive inputs over the postnatal period. Secondly, it will provide a rational basis for the use of reflex movements in the assessment of pain in hospitalised infants.

This introduction begins with an overview of the organisation of the cutaneous sensory system in the adult, with particular focus on the neuronal circuitry underlying the spinally mediated

flexor withdrawal reflex. Next, the postnatal development of sensory circuitry and the functional properties of nociceptive reflexes in early-life are described. Finally, a background to the clinical problem of pain management in the human infant is provided and current knowledge of the long-term effects of tissue injury and pain in early life are reviewed. This introduction serves as a foundation for the aims of the thesis. The aims are given at the end of this chapter.

1.1 Sensory processing is vital for survival

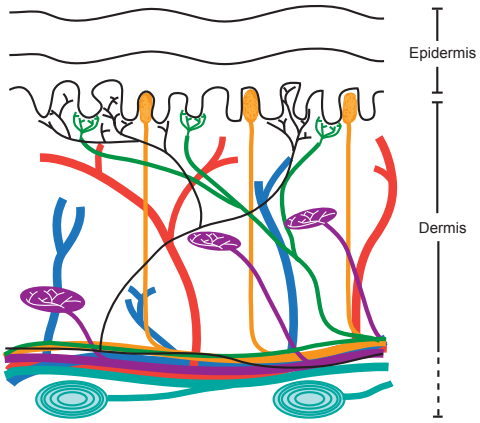
Sensory processing is vital for survival, an important function to provide information about the occurrence or threat of injury. The coding of cutaneous sensory information concerning tactile, thermal, painful and pruritic (itch) modalities from the skin (external) surroundings to the internal environment is complex. In terms of nociceptive processing, the system is capable of (1) detecting a diverse range of stimulus modalities, (2) distinguishing between noxious and innocuous events by setting specific sensory-response thresholds, (3) localising these events on the body surface (4) adapting to previous events by resetting sensory threshold and sensitising the system to protect against further injury.

Nociception is the neural process of sensory detection and encoding of noxious stimuli (Loeser *et al.*, 2008) which leads to the sensation of an unpleasant, sensory experience and the coordination of avoidance behaviour to prevent repeated contact with tissue-damaging stimulus. Four key phases are involved in this process: (1) '*sensory transduction*' (stimulus detection and conversion of stimulus energy into a neuronal signal); (2) '*sensory transmission*' (neural signalling from the periphery to the spinal cord); (3) '*synaptic transmission'* on to and within the spinal cord; (4) *'spinal cord output'* to the brainstem and higher centres for stimulus recognition and pain perception, accompanied by efferent projection to the motor system for reflex withdrawal.

1.1.1 Sensory transduction and peripheral transmission

The role of sensory transduction is to recognise the modality, intensity and duration, and location of the stimulus. The skin is the largest organ in the body and in an adult covers a surface area of approximately $2m^2$. Skin consists of an outer layer of stratified squamous epithelium – the epidermis, and an inner, thicker layer of connective tissue innervated by nerve bundles called the dermis. Sensory receptors located in the dermis convert external stimuli into neuronal signals. Primary afferent nerve fibres spread vertically across the dermis

and form horizontal neural plexuses below the epidermis, before terminating in the epidermal basement membrane as free-nerve endings (Figure 1-1). Sensory receptors are located on these free nerve endings. Sensory receptors are modality specific. For instance, thermoreceptors transduce temperature; mechanoreceptors detect mechanical input e.g. pressure, vibration, texture; and chemoreceptors respond to the presence of chemical compounds e.g. protons and histamine. Of particular interest in this thesis is the detection of mechanical and noxious stimuli.



Receptor type	Meissner corpuscule	Pacinian corpuscule	Ruffini's corpuscules	Merkel's discs	Free nerve endings
Function	Respond to vibration (20-40Hz)	Respond to vibration (150-300Hz)	Respond to pressure	Respond to pressure and vibration (5-15Hz)	Respond to mechanical, thermal or noxious
Location	Dermis	Deep layers of dermis	Dermis		Various

Figure 1-1: Schematic representation of mechanical sensory receptors in skin
Glabrous (non-hairy) skin, shown here, is innervated by numerous somatosensory neurons that encode mechanical and noxious stimuli (thermal not illustrated). Many neurons have complex endings that are associated with receptors that transduce physical stimuli. For example: Meissner corpuscles are mechanoreceptors and are optimally located close to the epidermis to encode light touch; Pacinian corpuscles are also mechanosensitive but respond to high pressure and are located in the deep layers of the dermis. Not to scale. Key to mechanical receptor type, function and location is given in the box. Arterioles are shown in red, venuoles in blue.

1.1.1.1 Mechanical stimulus

Tactile input is mediated by mechanoreceptors that are designed to respond to various types of physical stress such as pressure and vibration. There are four types of low-threshold mechanoreceptors: (1) Meissner's corpuscles, (2) Pacinian corpuscles, (3) Merkel's disks, and (4) Ruffini endings (Figure 1-1). Meissner and Pacinian corpuscles are rapidly adapting as they respond to the initial contact of a mechanical stimulus on the skin; Merkel's disks and Ruffini endings are slowly adapting and continue to fire during a constant mechanical stimulus. Meissner's corpuscles and Merkel's disks are located near the surface, at the

dermal-epidermal boundary and possess small receptive fields, whereas Pacinian corpuscles and Ruffini endings lie deeper within the dermis and have large receptive fields.

Since a standard mechanical stimulus commonly used in somatosensory research and used here in this thesis (Chapter 4; Study 2) is a set of calibrated von Frey hairs, the mechanism underlying the sensory transduction process following application of a von Frey hair is described below: -

A non-noxious mechanical (von-Frey hair) stimulus

Von Frey hairs are nylon hairs that exert a precise force when applied to the surface of the skin (see section 2.4.3 on page 65). The downward force, perpendicular to the skin surface, activates mechanoreceptors sensitive to pressure such as Merkel's corpuscles and Pacinian corpuscles. A small force will activate mechanoreceptors located superficially with sensitivity to low pressures. A strong force will activate low-threshold mechanoreceptors, as well as, high-threshold mechanoreceptors located deeper in the dermis. Despite intensive efforts to discover molecules that initiate touch sensation in mammals, the transduction mechanisms are largely unknown (see Lumpkin *et al.*, 2007 for review). It is thought that transduction channels are directly activated by mechanical stimuli although recently, transduction channels tethered to mechanically sensitive proteins have been implicated in mechanosensation, such as members of the *mec* gene family (Lumpkin *et al.*, 2007). Figure 1-2 illustrates the action of cutaneous von Frey hair application. The majority of fibre groups activated by low intensity von Frey stimuli will be the rapidly conducting, myelinated Aβ fibres, but some mechanoreceptors have smaller myelinated Aδ fibres. Importantly, the properties of these receptors are the same in mice, rats, cats, primates and man (Lewin *et al.*, 2004).

1.1.1.2 Noxious stimulus

Noxious input is mediated by nociceptors, a specialised class of sensory receptors that respond to levels of mechanical, thermal and chemical stimuli that are tissue threatening or tissue damaging such as noxious heat (>43^{o}C), intense pressure, irritant chemicals or sharp mechanical stimuli. To serve their protective function, nociceptors have a much higher threshold for activation than non-nociceptive receptors. Different subtypes of nociceptor exist and have been identified (Gold *et al.*, 2010; Julius *et al.*, 2001). Many nociceptors are polymodal, and respond to a broad range of noxious stimuli whilst others are more specific (Burgess *et al.*, 1967; Davis *et al.*, 1993). For instance, type I Aδ mechano-heat sensitive

nociceptors respond to pinch and slowly to heat >53°C, while type II respond to pinch and rapidly to heat >46°C. Similarly C-fibre nociceptors sensitive to heat, pinch or both have been identified (Craig, 2003).

Since the noxious stimulus used in this thesis is that of a heel lance, a common procedure performed in neonatal care to obtain a blood sample, the mechanism underlying the sensory transduction process following a heel lance is described below: -

A noxious mechanical (heel lance) stimulus

The lancet device comprises a rotating blade that rapidly incises the skin and blood vessels beneath. The stimulus application is comprised of two phases: (1) an initial tactile input from blade release and rotating movement through the lancet device, followed by (2) the noxious tissue-damaging injury (Figure 1-2). Phase 1 activates mechanoreceptors sensitive to vibration such as Meissner corpuscles and Merkel's discs. Phase 2 is the mechanical action of the blade through the dermis and activation of a range of high threshold mechanically sensitive nociceptors connected to Aδ and C fibres in the region.

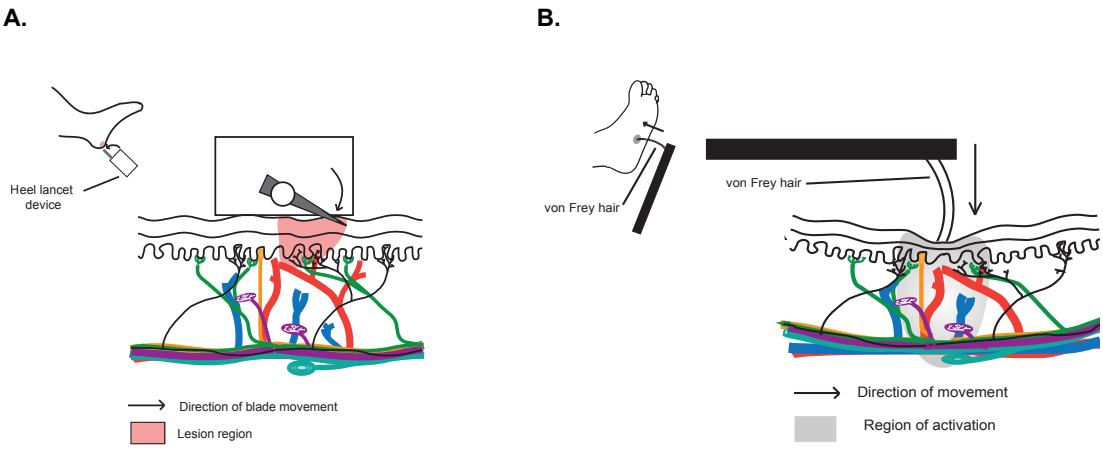


Figure 1-2: Schematic representation of cutaneous stimulation with (A) noxious heel lance tissue injury and (B) non-noxious mechanical stimulation
(A) Site of noxious heel lance stimulation of the infant heel; inset is a schematic representation of the site of skin incision by the release of the spring-loaded blade.
(B) Site of mechanical stimulation with SW monofilament to the surface of the foot; inset is a schematic representation of the site of cutaneous stimulation as the monofilament is applied to the surface of the skin. Downward movement of the monofilament creates stress through the dermis and activates mechanoreceptors. Not to scale (refer to Figure 1-1 for key).

Members of the transient receptor potential (TRP) ion channel family transduce physical and chemical stimuli into action potentials in nociceptor sensory nerves in the tissue. TRPV1, TRPM8 and TRPA1 are directly activated by heat, cold, chemical and mechanical force, and can also act as local integrators of multiple noxious stimuli (Stucky *et al.*, 2009). In addition, some sensory nerve terminals located directly in the incision will be severed which may trigger a barrage of primary afferent firing. The tissue damage caused by the lance will further activate nociceptors via the release of local inflammatory mediators. Inflammatory cascade components such as protons, and bradykinin, bind to ion channels or metabotropic receptors to activate second messenger signalling cascades respectively, and promote nociceptor sensitisation or prolonged activation (Julius *et al.*, 2001).

1.1.1.3 Stimulus intensity, duration and location

Activated sensory receptors initiate action potential firing along primary afferent fibres. The frequency of neural firing is determined by the relative intensity of the stimulus and type of receptor activated, and duration of firing dependent on stimulus presence and the type of sensory receptor activated. Most fibres maintain action potential firing, although an exception to these is the rapidly adapting mechanoreceptors - these respond exclusively to movement of the skin and not to static indentation. Meanwhile, those that respond to both movement and static indentation are called slowly adapting mechanoreceptors. As long as the stimulus moves, rapidly adapting neurons will not show a marked level of adaptation (Lewin *et al.*, 2004).

The cutaneous receptive field of a primary afferent neuron is the region of skin that it innervates and which, when stimulated, causes neural firing of its afferent fibre. Receptive fields overlap and so most cutaneous stimuli will activate many primary afferent neurons. Primary afferent neurons with receptive fields that are closest to the stimulation site are likely to fire at the highest frequency. Primary afferent fibres with receptive fields in the surrounding area will also fire but with lower frequency. Once the signal reaches the spinal cord, it is integrated with local circuit activity.

1.1.2.4 Peripheral nerve transmission

A-fibres

A-fibres have a fast conduction velocity due to their large diameter myelinated axons. There are three main classes of A-fibre: Aα, Aß and A∂. Fast conducting Aα and Aß fibres transmit

neural activity at speeds ranging between 35 and 120m/s and carry non-nociceptive information from the skin, muscle and joints to the spinal cord; A∂ fibres conduct at slower velocities between 5 and 35m/s and carry nociceptive mechanical, thermal and chemical information (Table 1-1).

C-fibres

C-fibres are much smaller and have a slow conduction velocity of 0.5-2.0m/s due to their small axon diameter and lack of myelination. Both noxious and innocuous information is transmitted along C-fibres to the central nervous system (Table 1-1). The majority of C-fibres are polymodal, responding to noxious, thermal and mechanical stimuli, others are mechanically insensitive but respond to noxious heat (Julius *et al.*, 2001). Silent nociceptors also exist and become activated following inflammation.

The type of primary afferent fibre activated will convey a particular sensation: A-fibre activation gives rise to a rapid first pain with a sharp, pricking-like sensation, while C-fibre activation triggers a delayed second, dull burning pain.

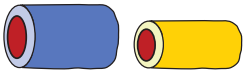
	Aα & Aβ fibres	Aδ fibre	C fibre
			
Physical characteristics	Large diameter: Aα, 13-20 um; Aβ, 6-12um Myelinated Conduction velocity: Aα, 80-120 m/s; Aβ, 35-75 m/s	Medium diameter: 1-5 um Myelinated Conduction velocity: 5-35 m/s	Small diameter: 0.2-1.5um Unmyelinated Conduction velocity: 0.5-2.0 m/s
Stimulus modality	Proprioception, light touch	Nociception (mechanical, thermal, chemical)	Nociception (mechanical, thermal, chemical) Innocuous temperature, itch
Pain type	----------	Fast, pricking pain	Slow, burning pain

Table 1-1: Properties of primary afferent fibres
Adapted from Julius et al (2001). Primary afferent fibres include small diameter (A∂) and medium- to large diameter (Aα, Aß) myelinated fibres, as well as small diameter unmyelinated fibres (C). Conduction velocity is directly related to fibre diameter. Most nociceptors are located on either A∂ or C-fibres and their different conduction velocities account for differences in pain sensation.

1.1.2 Spinal sensory processing: anatomy and physiology of dorsal and ventral horn circuitry

Primary afferent fibres transmit sensory information from the periphery to the dorsal horn of the spinal cord. Neural activity encoding stimulus modality and region of the body innervated is translated by the morphology and type of primary afferent fibre firing. Sensory input is processed at the spinal cord level by complex dorsal horn neuronal circuitry involving excitatory and inhibitory interneurons before transmission via projection neurons to supraspinal areas for further processing. Information from the dorsal horn also activates neural circuits in the spinal cord that are involved in the generation of local reflexes.

1.1.2.1 Dorsal horn lamina

Spinal cord grey matter is separated into the dorsal horn and ventral horn. There is a highly organised structure composed of layers (lamina) that are characterised by neural density and size as originally described by Rexed in the cat (Rexed, 1952). This structural arrangement is the same in other species including the human, monkey and rat. Primary afferents terminate in the dorsal horn with a specific distribution pattern according to their morphology. Figure 1-3 shows how each lamina receives input from distinct primary afferent fibre types.

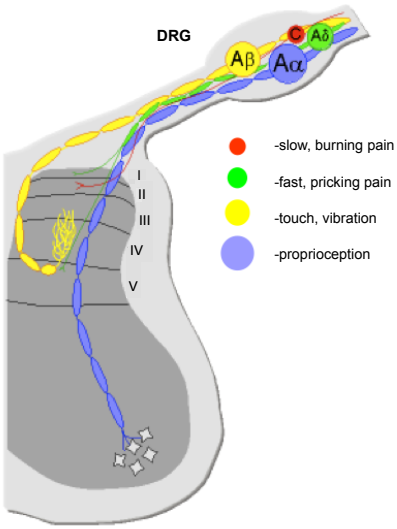


Figure 1-3: Schematic representation of dorsal horn organisation in the adult spinal cord
The spinal cord is composed of lamina characterised by neural density and size. The position of Rexed's laminae are shown by the horizontal black lines (laminae I located at the top-most part); laminae I-V constitute the dorsal horn. Primary afferent fibres terminate with a specific pattern; laminae I and II are the main target for nociceptive afferents. DRG, dorsal root ganglion.

Laminae I and II are the most superficially located and are the main target for nociceptive afferents, being innervated by nociceptive and thermoreceptive myelinated A∂ fibres and non-myelinated C-fibres. Lamina I is also referred to as the marginal zone, and Lamina II as the substantia gelatinosa due to the gelatinous appearance from the very low density of myelinated fibres. Together, Lamina I and II are known as the superficial dorsal horn. Lamina III – V, are located deeper in the dorsal horn and contain Aß terminals that encode low-threshold stimulation, as well as some A∂ and C-fibre nociceptive terminals (Todd *et al.*, 2006). Almost all lamina V neurons are wide dynamic range neurons, these respond to a range of tactile stimuli such as brush and pressure, and also to noxious heat, noxious cold as well as noxious inputs from the viscera and deeper tissues (Craig, 2003). Such multifaceted activity provides a representation of mechanoreceptive, proprioceptive and noxious input to the spinal cord. Laminae IV-VII and the ventral horn contain Aα fibre terminals arising from muscle spindles and Golgi tendon organs and are involved in proprioception (Hoheisel *et al.*, 1989).

1.1.2.2 Projection neurons

Projection neurons form a link between activity in the dorsal horn of the spinal cord and the brain. The axons of projection neurons traverse upward towards the thalamus along the contralateral spinothalamic tract, and to the medulla and brainstem via the spinoreticular/ spinoparabrachial (SRT) and spinomesencephalic (SMT) tracts (Figure 1-4). Thalamic nuclei process all somatosensory inputs to the central nervous system including processing sensory and discriminative aspects of pain. The lateral spinothalamic tract projects multimodal sensory inputs from wide-dynamic range neurons to the lateral thalamus. The medial spinothalamic tract and the spinoparabrachial tract project to the medial thalamus and limbic structures and are suggested to mediate the emotional-aversive components of pain (Tracey *et al.*, 2007). In this manner, nociceptive activity is integrated with homeostatic, arousal and autonomic processes, and transmitted to the forebrain regions for cognitive processing as well as involvement in the activation of the descending modulatory system.

The dorsal horn contains a precise arrangement of projection neurons. Lamina I contains a dense concentration of projection neurons, projection neurons are also prevalent throughout laminae III-IV but very few are present in lamina II at the lumbar level (Todd, 2010). Many projection neurons cross the midline of the spinal cord and travel in the contralateral white matter to the brainstem and thalamus. Histological studies of the rat lumbar cord show lamina I contains approximately 400 projection neurons (5% of the total population of lamina I

neurons); of these, 95% project to the lateral parabrachial area, 33% to the periaqueductal grey (PAG), 25% to the nucleus tractus solitaris and <5% to the thalamus (Spike *et al.*, 2003).

A large proportion (80%) of lamina I projection neurons express the neurokinin 1 (NK1) receptor for substance P, a neuropeptide which is released by nociceptive afferents and thus these cells respond to noxious stimulation (D'Mello *et al.*, 2008). Lamina 1 NK1-positive cells project to brain areas such as the thalamus, PAG and lateral parabrachial area (LPb) (Gauriau *et al.*, 2004). These cells also project to brainstem areas such as the rostral ventromedial medulla (RVM), a region that has descending projections to the dorsal horn (D'Mello *et al.*, 2008). Ultimately, lamina 1 NK1-expressing cells are important in modulating spinal processing by activation of descending pathways from the brainstem. Deeper in the dorsal horn, Lamina III and IV contain far less projection neurons that project predominantly to the thalamus and constitute a significant proportion of the spinothalamic tract (D'Mello *et al.*, 2008). Our knowledge about the neural circuits in these deeper laminae is much less comprehensive.

1.1.2.3 Interneurons

Interneurons act locally and mediate transmission of information at segmental levels of the spinal cord. For example, they are responsible for relaying commands from the superficial dorsal horn to ventrally located motor neurons to mediate nociceptive withdrawal reflex activity. Dorsal horn laminae I-III are densely packed with interneurons, with dendritic terminations located locally by extending a few segments rostrally or caudally in Lissauer's tract (the dorsolateral fasiculus) before re-entering the superficial dorsal horn (Szentagothai, 1964). Many interneurons remain in the same laminae but it is also common for interneurons to extend into other laminae.

Interneuron synaptic transmission is excitatory, mediated by glutamate release, or inhibitory, via GABA (γ-aminobutyric acid) and/or glycine release (Julius *et al.*, 2001). Glutamate is an excitatory neurotransmitter and activates two types of receptor: ionotrophic (AMPA, NMDA and kainate) and the G-protein couple metabotrophic receptor family (mGluR) receptors in the spinal cord (D'Mello *et al.*, 2008). Although there are no reliable immunocytochemical markers for the cell bodies of glutamatergic neurons to precisely identify glutamatergic neuron distribution, ionotropic glutamate receptors have been identified in the superficial dorsal horn (lamina I-II) and metabotropic glutamate receptors throughout the dorsal horn (Todd, 2010; Todd *et al.*, 2006). Glutamate-containing neurons are also expressed in laminae

I-III. Inhibitory GABAergic neurons and axon terminals are present in high numbers throughout the dorsal horn (see Todd, 2010 for review). In the rat, the distribution of GABA is approximately 25-30% of neurons in laminae I-II, and 40% of neurons in lamina III (Polgar *et al.*, 2003). Most of the GABAergic neurons in lamina I-III also express glycine and suggest that the majority of inhibitory interneurons either co-release GABA and glycine or just release GABA (Todd, 2010).

In lamina I, three main interneuron cell types have been described: pyramidal, fusiform and multi-polar. Lamina II interneurons can be separated into four main cell types identifiable by their morphology and neurotransmitter. Islet and central interneurons extend in a rostrocaudal direction and are elongated (>400μm and <400μm respectively); radial interneurons spread out in all directions but with small dendritic trees; vertical interneurons extend ventrally. Whilst radial and vertical interneurons are excitatory and release glutamate, islet cells are GABAergic and central cells utilise multiple neurotransmitters (Todd, 2010).

1.1.2.4 Descending pathways

Descending pathways modulate the excitability of spinal nociceptive networks and play a key role in the reaction to pain (Basbaum *et al.*, 1979; Fields *et al.*, 1977). Descending projections terminate diffusely throughout the dorsal horn and serve to modulate the balance of excitation and inhibition via synaptic input to primary afferent terminals, excitatory interneurons, inhibitory interneurons, projection neurons, and other descending pathway terminals. Sherrington was one of the first to demonstrate the influence of supraspinal modulation on spinal cord circuits; he showed that an increase in the flexion withdrawal reflex activity was observed after spinalisation in the decerebrate cat (Sherrington, 1910). Descending pathways can either inhibit or facilitate nociceptive processing (see section 1.1.3.1). The role of descending modulation in the regulation of pain is important for example in increasing inhibitory tone to encourage immobility and promote recovery following injury, whilst facilitatory input can serve to promote coordinated escape behaviour.

There are three main descending pathways involved in nociceptive regulation; a serotonergic pathway originating from the nucleus raphe magnus located in the medulla, a noradrenergic-mediated pathway originating from the locus coeruleus and other pontine regions, and a GABAergic pathway originating from the rostral ventral medulla (RVM). Serotonergic axons and noradrenergic axons are widely distributed throughout the dorsal horn but terminate most numerously in the superficial dorsal horn (Ruda *et al.*, 1982; Westlund *et al.*, 1980).

Likewise, GABAergic axons are also widely distributed in the dorsal horn and but synapse with lamina II interneurons (Todd, 2010).

1.1.2.5 Ventral horn components

Lamina IX of the ventral-lateral dorsal horn contains pools of motor neurons that project axons to individual muscles for the control of skeletal muscle movement and generation of flexion withdrawal (Woolf *et al.*, 1984). These are also referred to as alpha motor neurons. Motor neuron populations are organised in a columnar fashion rostrocaudally down the spinal cord in clusters arranged somatotopically (Romanes, 1951; Swett *et al.*, 1986). Despite extensive arborisation of motor neuron dendrites that can spread upwards as far as the base of the dorsal horn, primary afferent fibre terminals communicate with motor neurons by converging synaptically with reflex-encoding interneurons (Cook *et al.*, 1985; Schouenborg *et al.*, 1995b). Reflex-encoding interneurons are responsible for the coordination of target muscle groups and regulate reflex strength (Levinsson *et al.*, 2002; Schouenborg *et al.*, 1995a).

1.1.3 Anatomy and physiology of sensory and nociceptive pathways in the brainstem and higher-centres

This section aims to give an overview of the ascending and descending pathways from the spinal cord to the brain and how sensory information is processed at this level i.e. translation of nociception into pain perception. As mentioned previously, projection neurons are responsible for the neurotransmission of sensory information to the brainstem and higher centres for further processing. These include direct projections to the thalamus, homeostatic control regions in the medulla and brainstem, and projections to the hypothalamus and forebrain. Descending projections are responsible for modulation of spinal cord activity and include tracts from arising in the PAG of the brainstem.

1.1.3.1 Brainstem circuits

The thalamus and brainstem receive input from the spinal cord via projection neurons (see section 1.1.2.2 on page 28), and are key components of somatosensory processing including sensation and motor control. The thalamus is responsible for relaying somatosensory information to the primary sensory area of the cerebral cortex, as well as information about motor behaviour to the motor areas of the cortex. It represents the main relay structure for information to be transmitted to and from the cortex. The brainstem is particularly important

for integrating somatosensory activity with homeostatic, arousal and autonomic processes, as well as relaying information to the forebrain regions after brainstem processing. Spinal cord projections to the caudal ventrolateral medulla (CVLM) and nucleus tractus solitarius (NTS) are responsible for the integration of nociceptive and cardiovascular responses and have a role in the reflex tachycardia that results from a noxious stimulus. Projection neurons also synapse on to the parabrachial area which relays nociceptive information to the hypothalamus and limbic forebrain including the amygdala, anterior cingulate cortex and insular cortex, which are involved in processing the emotional aspects of the pain response, as well as autonomic response (Fields *et al.*, 2006).

Spinal cord activity and brainstem descending pathways coordinate together to maintain homeostasis. Descending pathways play a key role in the modulation of pain. The rostral ventral medulla (RVM) forms an endogenous control system with the PAG and dorsal horn of the spinal cord (Fields *et al.*, 2006). The RVM contains distinct classes of cells that inhibit and facilitate nociception. RVM ON cells show an increase in activity associated with nociceptive reflexes and the facilitation of nociception (Fields *et al.*, 1985). Whilst OFF cells show a pause in activity associated with nociceptive reflexes, and activation of these cells produces antinociception. PAG neurons make contact with RVM neurons projecting to the spinal cord, and modulate nociception by directly inhibiting and exciting ON and OFF cells in the RVM (Morgan *et al.*, 2008).

Cranial nerves V and VII, the trigeminal and facial nerve respectively, are implicated in the motor aspects of facial expression associated with pain behaviour. The anatomy of these structures is described later in section 1.1.5.

1.1.3.2 Transmission to higher centres; thalamus and cortex

There are several cortical and sub-cortical structures that are commonly activated by nociceptive stimulation including the anterior cingulate cortex, insular cortex, frontal and pre-frontal cortices, primary and secondary somatosensory cortices, thalamus, basal ganglia, cerebellum, amygdala, hippocampus and regions within the parietal and temporal cortices (Tracey, 2005).

Projection neurons predominantly terminate at the thalamus, a major relay centre for projection to the somatosensory cortex and the insular. The lateral thalamus is involved in the sensory-discriminative aspects and the medial thalamus involved in the affective-motivational

aspects of pain. Ascending projections from distinct lamina terminate on targeted thalamic areas (refer to section 1.1.2.2). Lamina I neurons convey nociceptive, thermal, visceral and muscular sensation and project to the posterior portion of the ventromedial nucleus of the thalamus (VMpo) which relays this information to the insular cortex, lamina I projections also terminate on the ventral caudal part of the mediodorsal nucleus of the thalamus (MDvc), which relays information to the anterior cingulate cortex, involved in affective and arousal component of the pain experience (Tracey *et al.*, 2007).

From the thalamus, information is transmitted along via higher order neurons to the cortex. Thalamocortical afferents project to the primary somatosensory cortex where sensory information from the contralateral body surface is mapped somatotopically i.e. with legs represented medially and face represented laterally (Penfield *et al.*, 1954). Projection to the primary somatosensory cortex is necessary for the sensory-discriminative aspects of pain processing e.g. localisation, intensity, temporal profile, quality (Dostrovsky *et al.*, 2006). The conscious experience of pain depends upon a combination of physical, emotional and cognitive factors (Tracey *et al.*, 2007). There is not a single pain centre in the cortex, rather a combination of brain regions that may be activated to generate the pain experience. Many brain regions play distinct roles in processing the pain experience and are collectively termed the 'pain matrix'. The pain matrix can be separated into a lateral (sensory-discriminative) and medial (affective-cognitive) pain pathway. Activation of neural circuits within the anterior cingulate cortex, insular cortex, secondary somatosensory cortex and possibly the thalamus are required to generate the perception of pain (Tracey *et al.*, 2007).

A. **B.**

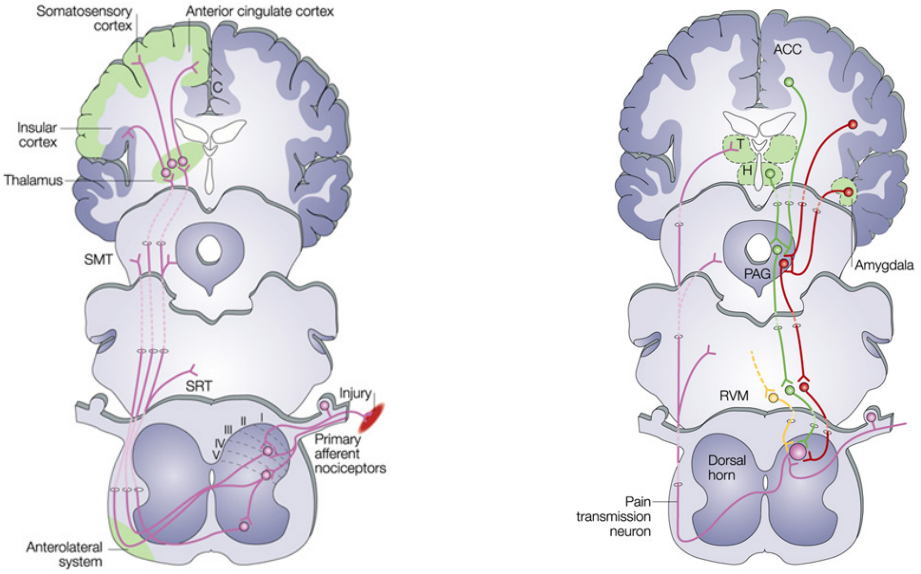


Figure 1-4: Ascending and descending pathways

Adapted from (Fields, 2004) (A) Primary afferent fibres from the periphery enter the dorsal horn of the spinal cord. Projection neurons from the dorsal horn cross to the contralateral anterolateral quadrant to the brainstem and thalamus (T), which contain higher order neurons that project to various cortical regions that mediate different aspects of the pain experience. These regions include somatosensory, anterior cingulate and insular cortices. SMT, spinomesencephalic tract; SRT, spinoreticular tract. (B) Descending nociceptive pathways: Limbic forebrain areas, including the anterior cingulate cortex (ACC), other frontal cortical areas, the hypothalamus (H) and central nucleus of the amygdala project to the midbrain periaqueductal grey (PAG), which in turn, indirectly controls pain transmission in the dorsal horn through the rostral ventromedial medulla (RVM). This pathway can exert both inhibitory (green) and facilitatory (red) control. A separate control channel through serotonergic neurons in the RVM (yellow) can also modulate pain in a state-dependent manner.

1.1.4 Spinal sensorimotor output: the flexion withdrawal reflex

Spinal cord projections transmit nociceptive information to supraspinal regions for the sensory-discriminative and emotive aspects of the sensory experience. Meanwhile local spinal cord circuits are involved in the coordination of reflexes, and can be modified by descending controls from supraspinal regions including the PAG and RVM. The flexion withdrawal reflex has been an important model for the study of nociceptive processing in the developing and adult spinal cord, and is described in this section.

1.1.4.1 An overview

The flexion withdrawal reflex was first comprehensively characterised by Sherrington in 1910 in the adult cat (Sherrington, 1910). Sherrington investigated the stimulus intensity, reflex receptive field pattern and coordination of muscle contraction using electrical stimulation to elicit flexion withdrawal of the hind limb. The key findings of this study formed a platform for our understanding of the flexion withdrawal reflex and were as follows:

(1) Stimulus intensity:

Flexion withdrawal reflex activity was more readily evoked at noxious levels of intensity than non-noxious levels; an increase in stimulus intensity increased the sharpness of the reflex response.

(2) Reflex receptive fields:

A flexion receptive field is generally defined as a region of skin that elicits sensory neuron activation when stimulated at intensities greater than threshold to cause limb withdrawal. The feline hind limb exhibited large flexion receptive fields across the whole leg. Further, cutaneous stimulation of the foot provoked reflex activity more easily than other regions of the limb, suggesting graded sensitivity across the cutaneous surface of the limb.

(3) Muscle coordination:

Classic flexion withdrawal of the ipsilateral limb to move the limb towards the body was accompanied by a contralateral limb extension; most hind limb muscles participated in withdrawal activity irrespective of stimulus location.

1.1.4.2 Stimulus intensity and receptive field properties

Sherrington's detailed observations of flexion withdrawal reflex activity eventually led to the proposal by Woolf & Swett that examination of the output of the spinal cord would help explain the neural circuitry underlying nociceptive processing between primary afferent terminals in the dorsal horn and flexor motor neurons and assist in the understanding of sensory mechanisms in the dorsal horn (Woolf *et al.*, 1984).

Subsequently, Woolf & Swett examined the single motor neuron responses in flexion reflex activity to distinguish stimulus modality, primary afferent fibre function and receptive field location in a comprehensive set of electrophysiological studies of the decerebrate rat. There were three main findings. Firstly, single motorneurons responded to high intensity mechanical stimulation of the ipsilateral foot (i.e. with firm pressure or pinch), high-intensity thermal stimuli (>49^{o}C and <10^{o}C), and the chemical irritant mustard oil; but not to light touch, brush, vibration or innocuous thermal stimuli (Woolf *et al.*, 1984). These results indicated that high-intensity polymodal (mechanical, thermal and chemical) input was characteristic of the flexion receptive field. Secondly, specific electrical stimulation of Aβ, Aδ and C-fibre afferents indicated that the reflex was evoked from stimulation of all these primary afferent types. Further, marked differences in the onset latency and duration of reflex responses were dependent on afferent fibre type stimulated; larger fibres elicited short latency reflexes due to the fast conduction velocity, while Aδ fibres elicited a short after-stimulus discharge, and C-fibre activation elicited activity with a long lasting after-stimulus discharge. Finally, the receptive field was found not to comprise a single very large sensitive area covering the whole limb (as originally suggested by Sherrington), rather the field size for each flexor motor neuron was variable ranging from a small field occupying part of the foot, to very large extending over the whole limb.

Studies examining the properties of the flexion withdrawal reflex in man concur with Sherrington, and Woolf and Swett's findings. In early human studies, noxious mechanical stimuli consisting of a needle piercing the skin which evoked a widespread distribution of muscle recruitment to enable flexion withdrawal, with a response amplitude that correlated with stimulus intensity (Kugelberg *et al.*, 1960). The same authors also confirmed that the flexion withdrawal reflex can be elicited with electrical and mechanical stimulation at intensities that correspond to both A and C-fibres involvement (Kugelberg, 1948), and later work established that these stimuli intensities were associated with the sensation of intense pain and burning (Willer, 1977). Further work by Hugon in 1973 indicated that the A-fibre

component contributed to an early phase of reflex activity termed RII, mediated by fast-conducting Aβ afferent fibres; this non-noxious RII component is not reliably observed across every subject. A late phase is mediated by nociceptive Aδ primary afferent fibres, and related to actual flexor withdrawal, followed by C-fibre contribution (see Sandrini *et al.*, 2005 for review). Electromyographic analysis of muscle activity confirmed that the distribution of cutaneous sensitivity for flexion receptive fields is heterogeneous when electrical stimuli were applied to various sites on the skin of lower limb (Hagbarth, 1960).

1.1.4.3 Flexion withdrawal muscle coordination consists of whole limb movement

Noxious stimulation leads to a precise withdrawal of the stimulated area away from the site of danger. The muscles recruited for limb withdrawal are dependent on the location where the stimulus is applied, so that the movement is directed away from the source of noxious input. The motor components of nociceptive withdrawal reflex circuitry are proposed to be organised in a modular fashion (Schouenborg, 2002). Every muscle has a unique cutaneous flexion reflex receptive field; with the muscle and receptive field building a module (or functional unit) so that the collaborative action of many activated reflex modules generates efficient coordination of the limb. This was first illustrated in the rat (Schouenborg *et al.*, 1992) and then the cat (Levinsson *et al.*, 1999a), and has since been demonstrated in the adult human (Andersen *et al.*, 1999; Sonnenborg *et al.*, 2000).

To maintain control of the reflex, different afferent fibre groups, both excitatory and inhibitory, converge onto common interneurons in the spinal reflex arc. The necessary reflex strength and motor neuron recruitment is coordinated by specific interneurons, termed 'reflex encoders' located in the deep dorsal horn (see section 1.1.2.3). Inhibitory reflex modules also exist and are located adjacently or overlapping with the excitatory receptive fields (Weng *et al.*, 1996). Activation of inhibitory receptive fields ensures inappropriate reflex responses are avoided. Together, these receptive fields are mapped in a highly functional manner to ensure optimal coordinated limb withdrawal accompanied with maintenance of suitable weight distribution across the limbs during rapid movement.

1.1.4.4 Supraspinal modulation of flexion withdrawal reflex activity

The nociceptive withdrawal reflex is a polysynaptic spinal reflex under the influence of supraspinal modulation. Nociceptive withdrawal reflex activity can be evoked in the absence

of higher brain function as originally demonstrated by Sherrington in the decerebrate, spinalised cat (Sherrington, 1910). Spinalisation elicits a state of sensitisation and hyperactivity, suggesting that the reflex is predominantly spinally mediated with additional tonic modulation at a supraspinal level. Spinalisation in the rat also leads to a reduction in cutaneous sensory thresholds and expansion of the reflex receptive field size (Schouenborg *et al.*, 1992; Woolf, 1984). In spinal cord injury patients where connections between the brainstem and spinal cord are impaired, nociceptive withdrawal activity is characterized by expansive reflex receptive fields (Andersen *et al.*, 2004; Grimby, 1963b; Kugelberg *et al.*, 1960; Schmit *et al.*, 2003) and increased reflex activity (Dimitrijević *et al.*, 1970). Further, brainstem descending projections and spinal cord activity can be modulated by morphine administration to reinforce inhibition (anti-nociception). Willer and colleagues (1980) administered intravenous morphine to healthy adult subjects and showed that opioid-mediated supraspinal inhibition depresses the magnitude of flexion withdrawal reflex activity when elicited by a constant (electrical) stimulus intensity. These studies also showed that higher doses of morphine exerted a greater inhibitory effect and could be reversed by naloxone administration. Collectively, Willer's work indicated that mechanisms of morphine-induced analgesia involved a direct depressive effect on nociceptive transmission in the spinal cord. (Willer *et al.*, 1980).

1.1.4.5 Flexion withdrawal reflex activity and pain perception

A strong correlation between reflex magnitude, stimulus intensity and subjective pain magnitude ratings has been shown in numerous human studies (e.g. Bromm *et al.*, 1980; Chan *et al.*, 1989; Dowman, 1991; Willer, 1977). Willer utilised electrical stimulus to evoke reflexes whilst measuring electromyographic (EMG) activity in the biceps femoris, in combination with a verbal report scale to clearly demonstrate that the threshold required to evoke a flexion withdrawal reflex is equivalent to that required for pain sensation in adult man (Willer, 1977).

Nociceptive reflex withdrawal activity and pain perception are both altered by pharmacological interventions known to affect pain sensitivity. Pharmacological studies have shown that morphine administration dampens the magnitude of reflex activity in a dose-dependent manner, illustrating the depressive effects of opiates on nociceptive transmission at the spinal level (Willer, 1985; Willer *et al.*, 1980). Furthermore, administration of ketamine (a glutamate receptor antagonist) inhibits central sensitisation induced by high-intensity

repetitive stimulation- as observed by dampened flexion withdrawal reflex activity and pain perception in a placebo-controlled study (Arendt-Nielsen *et al.*, 1995).

The flexion withdrawal reflex is influenced by psychological factors including distraction and anxiety, and by clinical pain states such as neuropathic and inflammatory pain, migraine and irritable bowel syndrome (Latremoliere *et al.*, 2009; Sandrini *et al.*, 2005). Willer *et al* found that human subjects whose attention was diverted away from a noxious stimulus by performing a calculation exhibited a decrease in flexion withdrawal reflex activity suggesting the influence of higher structures on spinal nociceptive processing (Willer *et al.*, 1979).

Recent work by Neziri and coworkers has investigated pain perception and reflex threshold values following electrical stimulation in a large study of 300 adult males and females (Neziri *et al.*, 2009a). The authors discuss the potential for using these data as an indicator of normative values for comparison in pharmacological and physiological studies of nociceptive processing. Collectively the results of these studies confirm that reflex activity and subjective pain rating increase linearly with increasing stimulus intensity, supporting the validity of the flexion withdrawal reflex as an objective measure of nociceptive processing.

1.1.4.6 Central sensitisation of reflex activity

Tissue injury leads to reduced sensory thresholds, expansion of reflex receptive fields and exaggerated reflex activity to additional stimulation. This is due to a combination of peripheral mechanisms such as the release of inflammatory mediators which sensitise cutaneous sensory receptors close to the site of injury (Julius *et al.*, 2001) and changes in central neural circuitry termed 'central sensitisation' (Woolf, 1983). Central sensitisation is an important neurophysiological phenomenon that protects the organism from further damage through sensitisation of the central nociceptive system (see Latremoliere *et al.*, 2009 for review). These centrally mediated changes are characterised by abnormal responsiveness to noxious and innocuous stimuli and a spread of tenderness away from the site of injury; they are typically found following tissue injury and in many clinical pain states. Central sensitisation can be induced experimentally in a naïve organism by applying repeated stimuli of constant intensity (Price, 1972). Increases in the size of reflex receptive field and magnitude of reflex response have also been reported in healthy adults in response to repeated focal painful stimuli (Spaich *et al.*, 2005). This change in nociceptive activity is not usually a permanent process and sensitivity typically returns to baseline levels over time. As changes in

plasticity in the central nervous system correlate well with alterations in the flexion withdrawal reflex, the reflex can be useful as a measure of central nociceptive processing.

1.1.4.7 Flexion withdrawal reflex as an index of nociceptive activity

The flexion withdrawal reflex has been used in numerous laboratory studies as a measurement of spinal nociceptive processing and central excitability of nociceptive circuits. Although nociceptive withdrawal reflexes are not direct evidence of pain perception, they provide robust information about the sensitivity of the central nervous system to noxious stimuli. The previous section on flexion withdrawal reflex activity has established that it is a useful index of nociceptive activity by indicating the following:

(1) A noxious stimulus will evoke flexion withdrawal reflex activity
(2) Flexion withdrawal reflex activity consists of movement of the whole limb and can be easily measured using non-invasive techniques
(3) Flexion withdrawal reflex activity is modulated by supraspinal projections on to the spinal cord
(4) Flexion withdrawal reflex activity is correlated with pain perception
(5) Flexion withdrawal reflex activity is affected in clinical pain states due to altered modulation of nociceptive circuitry

1.1.5 Other types of physiological activity used as a measure of nociceptive processing

Other pain responses including facial expression, and alterations in heart rate and respiratory rate are also used as indicators in clinical pain assessment (see section 1.3.2, page 54). Of particular interest is facial expression, the relationship of which with flexion withdrawal reflex activity will be investigated in Study 1 and Study 3 later in the thesis. The components and circuits underlying facial motor activity will be briefly discussed in this section.

1.1.5.1 Components of facial expression

Facial expression is important in communicating distress to others and has been extensively studied in adults (Williams, 2002). From birth, crying or facial expression is the primary form of communication due to the absence of speech. Facial movement is under voluntary control, however the limbic motor projections on facial motor nuclei control emotional expression and

thus strong emotions such as crying or wincing are typically involuntary. The highly reproducible nature of facial expression following a painful procedure has led to it's use as a key indicator in infant pain assessment (Grunau *et al.*, 1987). Interestingly, recent work by Mogil and colleagues has developed a facial coding system to gauge the degree of experimental pain experienced in the laboratory mouse (Langford *et al.*, 2010). Several striking similarities in facial pain coding features exist between the mouse and human (at infant and adult ages); these include eye squeezing, and nasolabial furrow or 'cheek bulging' (appearance of a fold of skin between the cheek and corner of the mouth or of cheek muscle between nose and eye in the mouse) indicating an evolutionarily conserved mechanism (Langford *et al.*, 2010; Prkachin, 2009).

1.1.5.2 Anatomy of trigeminal (V) and facial (VII) cranial nerves

There are twelve pairs of cranial nerves, most of which innervate the head and neck. Trigeminal and facial cranial nerves are responsible for the coordination of facial expression and are located in the brainstem. There is a close anatomical and functional relationship in the motor divisions of these two nerves.

The trigeminal nerve is the largest cranial nerve (V) and is the sensory nerve to the greater part of the face, and the motor nerve to several muscles including those involved in mastication (Snell, 2001). There are three main branches, the ophthalmic nerve, the maxillary nerve and the mandibular nerve. The former two serve as sensory afferents whilst the latter is comprised of both sensory and motor axons. The motor nucleus of the trigeminal nerve receives bilateral corticonuclear input from both cerebral hemispheres and innervates the muscles of mastication. Lesions to the trigeminal nuclei lead to jaw tremor, involuntary chewing or trismus (clenching of teeth) (Sanders, 2010).

The primary motor nucleus of the facial nerve (VII) is located within the reticular formation of the pons. The facial nerve innervates the muscles of the lower and upper part of the face for facial expression. The part of the nucleus that supplies muscles of the lower part of the face receives corticonuclear fibres from the contralateral cerebral hemisphere; the part of the nucleus that supplies the muscles of the upper part of the face receives corticonuclear fibres from both cerebral hemispheres (Snell, 2001). Lesions to the facial motor nerve are identified by facial paralysis; the corner of the mouth droops, creases and skin folds are effaced, the forehead becomes unfurrowed and eyelids will not close (Sanders, 2010).

1.1.6 Animal models provide information on the development of nociceptive processing

Research using animal models has significantly advanced our understanding of nociception and the development of the nervous system. Animal studies have allowed us to investigate the cellular and molecular mechanisms underlying the nociceptive withdrawal reflex in a manner that would be unfeasible in humans. For instance, acute and chronic pain can be simulated in animal using models through hind-paw incision or spared nerve injury, following which pharmaceutical compounds with known mechanisms of action can be tested for analgesic efficacy and toxicity. Furthermore, long-term developmental studies can be completed over a relatively short time frame of weeks to months rather than the many years required for a human to reach full maturity.

Animal models have been used extensively to provide information on the development of the nervous system. The rat, mouse and human genomes encode a similar number of genes (Gibbs *et al.*, 2004). The laboratory rat is a useful research model due to its genetic (and consequently) neurological similarity to humans, as well as its low maintenance cost and high reproductive turnover. The gestational period for a rat is 21.5 days and for a human is approximately 270 days (37-42 weeks). The first two postnatal months encompass the developmental period of the rat from neonate to adult; weaning age is postnatal day (P) 21 after which the rat undergoes sexual maturation, reaching adolescence at P35 and young adulthood at >P63 (Mccutcheon *et al.*, 2009). In terms of CNS development, the first 7-10 postnatal days (P7-10) in the rat are equivalent to a preterm human infant of 28 weeks gestational age (GA) (Fitzgerald *et al.*, 2009). Comparisons between rat-pup and neonatal nervous system anatomy and physiology reveal clear similarities in the development of nociceptive processing (Fitzgerald, 2005).

The use of animals in pain research remains a necessity, however it is important to translate findings from animal studies into a better understanding of human physiology and ultimately improved medical treatments (Mogil, 2009; Mogil *et al.*, 2010).

One key advantage of human research is qualitative analysis of the pain experience can be measured – humans can inform us of how much pain they are feeling whereas an animal cannot. Pain is necessarily assessed in the animal using behavioural measurements that are subjective in nature and may not reflect the actual pain experience. These limitations are not confined to pain assessment in animals, indeed studies in non-verbal humans including

neonatal, comatose and demented patients, often use subjective measures to estimate the degree of pain experienced (see section 1.3.2 on page 54).

1.2 The development of nociceptive circuits

In the animal and human developing nervous system, the neuronal circuitry underlying the flexion withdrawal reflex is immature and subject to substantial refinement in early life. The neonatal spinal reflex pathway is highly excitable and as a result the following physiological characteristics prevail: (1) cutaneous sensory thresholds are lower, (2) receptive fields exhibit uniform sensitivity, and (3) muscle coordination is inappropriately directed with more synchronised and long-lasting contractions (Fitzgerald, 2005; Fitzgerald *et al.*, 2009). The development of nociceptive circuitry and the neuroanatomy and neurophysiology underlying these findings are described in more detail below.

1.2.1 Developmental characteristics of flexion withdrawal reflexes

(1) Sensory thresholds are low and increase with age

Flexion withdrawal reflex activity is readily evoked by non-noxious as well as noxious stimulation in the neonatal animal and human infant; it is not a specific reflex as in the adult. Behavioural studies show that rat pups exhibit low cutaneous sensory thresholds, with hind-limb withdrawal reflex responses evoked following weak intensities of thermal and mechanical stimulation that are innocuous in strength (Fitzgerald *et al.*, 1988b; Holmberg *et al.*, 1996). Likewise, human infants have low flexion reflex thresholds, reflected by the small cutaneous force necessary to evoke flexion withdrawal (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b); e.g. from 0.52g at 30-35 weeks GA to 1.7g at 39-44 weeks GA (Andrews, 1997).

Adult flexion reflex thresholds are much higher than in the neonate, and developmental studies have shown that flexion reflex thresholds increase over the postnatal period to become more adult-like by P14 in the rat pup (Holmberg *et al.*, 1996). Flexion reflex thresholds in the human rise with gestational age (GA) (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b), and this is independent of postnatal age in human infants as evidenced by an investigation of sensory thresholds in preterm infants studied at full-term that were equivalent to normal full-term infants (Fitzgerald *et al.*, 1988b).

The increased reflex excitability associated with early development may be due to decreased skin thickness, increased responsiveness of primary afferent fibres, altered dorsal horn neurotransmission or changes in ventral horn processing. However, the components of the peripheral system are well-developed by birth as shown by mechanoreceptor activity in the rat pup, which consists of the same response patterns and sensory thresholds seen in the adult (Fitzgerald, 1987a). Likewise, efferent output underlying the motor components of the stretch reflex, which uses the same set of ventral horn motor neurons as those in hind-limb withdrawal, are functional in the rat from P0 (Kudo *et al.*, 1985). Collectively these findings suggest that delayed maturation of the central processing components is most likely to be responsible for changes in cutaneous sensitivity.

Repeated innocuous stimulation can evoke sensitisation in the neonate. In very young human infants, repeated stimulation of the foot at intervals of 10s or less results in a sensitisation of the reflex response, characterised by an increased force of withdrawal activity with rhythmic flexor and extensor movement and increased frequency of responses; this is seen in infants up to approximately 37 weeks GA after which they respond with increasingly dampened or absent reflex activity with increasing age (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). In healthy adults and spinal cord injury patients, repetitive stimulation leads to a decrease in flexion withdrawal reflex activity that is independent of stimulus type, electrical or mechanical, and an intact spinal cord (Dimitrijevic *et al.*, 1972; Dimitrijević *et al.*, 1970; Dimitrijević *et al.*, 1971) further supporting the suggestion the importance of central processing in modulating the nociceptive circuitry.

(2) Receptive fields are uniform in sensitivity distribution

In the human infant, the reflex receptive field is homogenous and encompasses the whole limb, from the toes to the top of the thigh and buttock (Andrews *et al.*, 1994). Stimulation within this large receptive field at intensities greater than threshold will evoke withdrawal of the limb. In the adult, the sensitivity distribution across the reflex receptive field is not homogenous (with maximal sensitivity observed on the sole of the foot and minimal sensitivity at the top of the leg) (Andersen *et al.*, 2004; Neziri *et al.*, 2009b; Sonnenborg *et al.*, 2000), a far more uniform distribution of cutaneous sensitivity across the reflex receptive field is observed in the rat pup (Holmberg *et al.*, 1996) and human infant (Andrews *et al.*, 1994). Developmental studies show that sensitivity distribution across the flexion reflex receptive field becomes more defined with increasing age, reaching an adult-like distribution in the rat in the third post-natal week (P20-25) (Holmberg *et al.*, 1996) and at least switching toward

heterogeneity after 37 weeks GA in the human infant (Andrews *et al.*, 1994). This could be due to changes strengthening the inhibitory circuitry in the developing dorsal horn (Baccei *et al.*, 2004; Bremner *et al.*, 2006) and changes in the organisation of dorsal horn cutaneous receptive fields (Bremner *et al.*, 2008).

(3) Muscle coordination is imprecise and poorly directed

Neonates are capable of mounting bodily responses to sensory stimuli. However, cutaneous reflexes in the newborn consist of exaggerated responses, with synchronous movement of multiple limbs and are poorly directed relative to the stimulation site (Fitzgerald, 2005). Such reflex activity is distinct from the adult response that directs the single stimulated limb away from the region of injury in an efficient and coordinated fashion.

In the neonate, exaggerated reflex responses are associated with long lasting muscle contractions. EMG studies of hind-limb activity provide a quantitative measurement of the reflex response and have shown the duration of flexion reflex activity is prolonged in younger animals compared to the adult rat; in P1 rats, a single pinch above threshold force evokes motor reflex activity that lasts for up to 20s but by the third postnatal week the evoked response is just less than 1s (Fitzgerald *et al.*, 1984). This pattern of activity was also reflected with thermal and electrical stimulation (Fitzgerald *et al.*, 1984).

Uncoordinated movement of all limbs has been reported using behavioural observations of withdrawal reflex activity in the kitten (Ekholm, 1967), rat-pup (Holmberg *et al.*, 1996) and human infant (Andrews *et al.*, 2002b; Franck, 1986). The gradual fine-tuning of the underlying reflex network is reflected by progressive refinement of reflex behaviour in the first three postnatal weeks in the rat (Holmberg *et al.*, 1996). O'Sullivan *et al* (1991) showed reflex radiation i.e. recruitment of muscles other than those stimulated, decreases with age in studies examining the stretch reflex of the upper limbs. Indeed, in the human reflex laterality is developmentally regulated and abdominally-evoked flexion withdrawal studies report a predominantly bilateral withdrawal is evoked up to 36 weeks GA, then ipsilateral withdrawal between 36 and 42 weeks GA followed by no limb withdrawal in older infants (Andrews *et al.*, 2002b).

The direction of reflex movement is unfocused at birth. Waldenstrom *et al* (2003) used thermal stimulation to one-side of the rat tail (using focused lasers) to show a high error rate

of tail-flick responses (with movement directed towards the stimulus) that occurs more frequently in the youngest rap-pups, between P0 to P10, and gradually improves over the first 3 postnatal weeks. Interestingly, the improvement in direction of tail movement was delayed if cutaneous tactile input was abolished by chronic application of local anaesthetic EMLA cream (Eutectic Mixture of Local Anaesthetic) demonstrating the importance of sensory experience in shaping neural networks (Waldenstrom *et al.*, 2003).

1.2.2 Developmental neuroanatomy and neurophysiology

At the time of birth, many of the basic sensory and motor connections are already established. The developing spinal cord undergoes extensive refinement of the neuronal circuitry underlying sensory processing to enable the functionally appropriate sensorimotor responses to noxious stimuli that are seen in the adult. Neurons undergo Hebbian learning wherein inputs to a given cell that are coincident with action potential firing undergo synaptic strengthening. Additionally, synaptic elimination takes place, a process of programmed cell death or apoptosis of neurons that do not form functional synapses in the immature nervous system. Other processes including axonal arborisation, myelination and tuning of synaptic kinetics are also contributory to the fine-tuning of the nervous system. Developmental neuroanatomy and physiology have been extensively studied using a plethora of histological, *in vitro* and *in vivo* techniques in the rat. However, our knowledge of human development is less clear. The development of nociceptive circuitry is discussed in the following section and primarily focuses on research from the rat (for comphrehensive review see Fitzgerald, 2005; Fitzgerald *et al.*, 1999). A summary of the comparison of the developmental characteristics between the rat and human are given at the end in Table 1-2 on page 53.

1.2.2.1 Reflex activity in the foetus

Prior to birth, *in utero* cutaneous sensation occurs from the 2nd week of gestation in the rat [embryonic day (E)15; the rat gestation period is 21.5 days] and by the 8th gestational week in the human (the human gestational period is 37-42 weeks). In both species the spread of sensitivity progresses in a dorsoventral direction. In the foetal rat, the perioral region is the first area of the body to become sensitive at E15, followed by the forepaws at E16, and the feet and end of the tail by E18-19 (Baccei *et al.*, 2006). In vivo recordings of rat foetal dorsal root ganglion (DRG) neurons between E16-20 show that sensory afferent fibres are very active and respond to specific cutaneous stimulation; whilst considerable spontaneous activity is present presumably to enable the appropriate formation of synaptic connections, afferent

fibres exhibit defined receptive fields and repetitive firing patterns to mechanical and thermal stimulation (Fitzgerald, 1987c). Likewise, in the human foetus at 10 weeks GA, local facial reflexes were reported when stimulating areas innervated by the trigeminal nerve with gentle (innocuous) stroking from a hair, followed by sensitivity of the hands and soles of the feet by 12 weeks GA (Humphrey, 1964). In summary, it is clear that the basic sensory and motor connections in the spinal cord are already established however considerable maturation takes place during the postnatal period in both rat and human.

1.2.2.2 Peripheral and central projection of primary afferent fibres

Primary afferent axons develop from the DRG and project to the periphery and to the dorsal horn before birth. The numbers of DRG neurons gradually increase, (with A-fibre neurons preceding C-fibre neurons) until before birth. In the rat, the overall number of cells decreases by 15% over the first 5 postnatal days coinciding with cutaneous innervation (Coggeshall *et al.*, 1994). The growth of DRG neurons into the periphery, formation of cutaneous terminals and structural organisation of dorsal horn neurons will be discussed below together with functional features of these sensory components:

(1) Peripheral axon growth & myelination

A- and C-fibre axon projections reach the rat hind limb from E13-14 and extend to the most distal part of the foot by birth a week later (Jackman *et al.*, 2000). Peripheral terminals of A and C fibres grow into the surface of the epidermis before withdrawing backwards to form a subepidermal nerve plexus, with A-fibres being the first to form a nerve plexus in the dermis which by the end of the foetal period is densely innervated (Jackman *et al.*, 2000; Reynolds *et al.*, 1991). Free nerve endings branch up into the epidermis to accompany this process (Reynolds *et al.*, 1991). Histological studies on the human foetus show that free nerve endings are distributed throughout the epidermis with a dense subepidermal nerve plexus from 26-28 weeks GA (Hewer, 1935).

In the rat, primary afferent axons are surrounded by a small number of myelinating and non-myelinating Schwann cells from E17 onwards. Myelinating Schwann cells progressively wrap around the axons in a slow process that is complete by the 3rd postnatal week (Mirsky *et al.*, 2002). Increased conduction velocity accompanies myelination and conduction velocity increases by 6-fold from birth to P14 in rat DRG neurons (Fulton, 1987). Cutaneous reflexes of the lower limb in the human reduce in latency to onset over a prolonged postnatal period, until 10 months of age (Vecchierini-Blineau *et al.*, 1982). The conduction velocity of the

tibial nerve in the neonate at 27 weeks is approximately 6m/s and increases to 26 m/s by 37 weeks (full-term); (Schulte *et al.*, 1968). Peripheral nerves reach maximum fibre diameter and conduction velocity at approximately 5 years of age (Eyre *et al.*, 1991).

(2) Cutaneous terminals

A-fibres have both mechanoreceptors and nociceptors at their nerve terminals (see section 1.1.1) and maturation of these terminals occurs at varying degrees pre-and postnatally in the rat. Merkel cells are functional at birth although Meissner corpuscules do not develop until a week later at P8 (Baccei *et al.*, 2006). By contrast, nociceptors develop from an early age; for instance, the TRPV1 receptor which is located on C-fibres and encodes noxious thermal and chemical stimuli is expressed in DRG neurons from P2 at levels akin to the adult, as is the purinergic P2X3 receptor which is responsible for thermal and mechanical hyperalgesia following inflammation or nerve injury at this time (Fitzgerald, 2005). In the human, histological studies on the cutaneous innervation of the human foetal finger indicate that the terminal formations of Pancinian corpuscules and Meissners corpuscules are formed by 16-20 weeks GA (Beckett *et al.*, 1956).

In vivo recordings of DRG neurons show cutaneous primary afferent activity in the rat pup, consists of the same response patterns and sensory thresholds seen in the adult (Fitzgerald, 1987a). In the rat, mechanical and thermal stimulation (mediated by A-fibres) evoke flexion withdrawal reflex activity from birth and show a functionally mature somatosensory system (Fitzgerald *et al.*, 1984). Mustard oil, which causes long-lasting firing of C-fibres and flexion reflex withdrawal in the adult (when applied to the paw), has no effect at birth and only by P10-11 are small reflex withdrawal responses observed (Fitzgerald *et al.*, 1984). These studies suggest that central rather than peripheral maturation are responsible for changes in dorsal horn neuron circuitry and spinal reflex excitability.

(3) Dorsal horn neurons

The spinal cord develops ventrodorsally, with deep dorsal horn neurons generated after motor neurons and the superficial dorsal horn neurons being the last to mature. Primary afferent fibres first grow from the DRG into the spinal cord at different times depending on fibre type; large diameter A-fibre afferents enter the grey matter at E15-17, followed by C-fibres from E19 onwards in the rat (Fitzgerald, 2005; Fitzgerald *et al.*, 1999). Both Aδ and C-fibres terminate in lamina II just prior to birth at E19.5-20 (Fitzgerald, 1987b). In younger rats,

lamina organisation of the primary afferent terminals in the dorsal horn is diffuse and suggested to exhibit a 'floating organisation'. Considerable reorganisation of primary afferent terminals takes place over the first 2 postnatal weeks until the morphology is becomes stratified and more adult-like (Granmo *et al.*, 2008). This is a consequence of synaptic elimination, pruning of inaccurate dendrites and the establishment of new axonal connections.

In humans, primary afferents enter the grey matter of the spinal cord at 10 weeks GA (Konstantinidou *et al.*, 1995). Between 11 and 19 weeks afferent fibres project to the ventral horn motorneuron pools, suggesting the formation of the monosynaptic stretch reflex circuitry at this stage. At the same time, other classes of primary afferents terminate in laminae III and IV with a striking paucity of superficial dorsal horn (lamina I-II) afferent terminal distribution over this period (Konstantinidou *et al.*, 1995).

Electrophysiological recordings from single cutaneous primary afferent units in the rat DRG show that strong responses to cutaneous mechanical stimulation are elicited at low stimulus intensities from birth. Initially, some cells respond to innocuous brushing and noxious pinching but the convergence of input changes over the postnatal period (Fitzgerald, 1987a). In young animals, between P3-P10, responses are mainly elicited from innocuous stimulation but by P21 convergence develops and the percentage of neurons with innocuous and noxious input is similar to that seen in the adult (Fitzgerald, 1987a). In the adult, noxious cutaneous or Aδ and C-fibre stimulation specifically induce *c-fos* expression. In the neonate however, innocuous or low-intensity stimuli evoke *c-fos* expression throughout the superficial dorsal horn (Jennings *et al.*, 1996). Also, cutaneous receptive fields are larger relative to the size of the paw at P3 rats than in the adult rat (Fitzgerald, 1985; Torsney *et al.*, 2002).

1.2.2.3 Ventral horn motor neurons

In the rat, motorneurons are produced from E11-13 (Nornes *et al.*, 1974). Whilst initially present in high numbers a 40-45% loss of motor neurons in the spinal cord occurs between E16 and birth (Oppenheim, 1986). This process of neurodegeneration ensures the formation of functionally appropriate connections to the muscle is made. Dendritic arborisation of motor neurons develops postnatally, although the number does not change, the dendrite branches gradually increase in length during the first 8 postnatal weeks. Age-related changes in electrophysiological properties of motorneurons also occur. Motor neurons are excitable from E15. Some early motor neurons communicate via electronic coupling though gap

junctions, though this gradually decreases until gap junctions are no longer present after the first postnatal week. Gap junctions are suggested to contribute to synchronous firing and play a role in shaping discharge patterns. At the early postnatal age, motor neurons are more excitable to synaptic inputs compared to adults, due to small motor neuron size and high input resistance (Vinay *et al.*, 2000). Therefore, a smaller current is needed to initiate cell firing, and this increased excitability may account for the abundance of spontaneous activity in spinal networks. Action potential properties change with age. Threshold increases with age, from -35mV at E15-16, to -47mV at P1-3. Action potential amplitude also increases whereas action potential duration decreases in neonatal motor neurons relative to perinatal motor neurons.

In the human, ventral horn motor neurons are present throughout the spinal cord from 11 weeks GA (Forger *et al.*, 1987). Motor neurons are clustered more densely in the youngest foetus (11 weeks GA) and by 25 weeks GA disperse into a distribution similar to that found in the adult spinal cord (Forger *et al.*, 1987). This is accompanied by a reduction in motor neuron number with gestational age, approximately 35% of motor neurons present at 11 weeks GA degenerate by 25 weeks GA (Forger *et al.*, 1987). Neuromuscular junctions first appear in the first 10-13 weeks GA in the human foetus; on the intercostal muscle at 11 weeks, at the tibialis at 12 weeks and biceps by week 13 GA (see Forger *et al.*, 1987).

In the neonate (rat and human) each muscle fibre is innervated by several different motor neuron axons (polyneuronal innervation), whereas in the adult only one axon innervates each muscle fibre (mononeuronal innervation). Synapse elimination refines neuron-motor connections and neurons that do not form functional synapses in the immature nervous system undergo programmed cell death or apoptosis. The transition from polyneuronal to mature mononeuronal innervation occurs over the first 2 postnatal weeks in the rat (Buffelli *et al.*, 2004). Elimination of polyneuronal innervation starts later in the soleous muscle (P8) than in the flexor muscles (P3) of the rat, and reflects a delay in the rat of maturation of motor pools innervating antagonistic muscles (Vinay *et al.*, 2000). Synapse elimination occurs in the periphery and involves motor nerve endings and axon branch retraction Each motor unit, which is comprised of a single α motor neuron and all of the muscle fibres it innervates goes from containing a great number of muscles fibres which receive overlapping input from many other motor neurons to containing a smaller number of muscle fibres with non-overlapping input in the mature system.

1.2.2.4 Descending control of spinal circuitry

Brainstem-spinal cord projections are important to regulate reflex withdrawal. In the rat, brainstem nuclei targeted for projection on the spinal cord are generated from E11-E15. First, projections reach the white matter of the cervical cord (at E13-14), followed by the thoracic cord (at E14-15) and the lumbar cord before birth (see Vinay *et al.*, 2000 for review). The earliest corticospinal projections arrive by the end of the 1st postnatal week. Despite these anatomical features, descending pathways to the dorsal horn are not functionally mature until P21 (Fitzgerald *et al.*, 1986). However, descending pathways are important for development since neonatal spinalisation leaves spinal reflexes permanently disorganised in adult rats (Fitzgerald *et al.*, 1986; Levinsson *et al.*, 1999b) Connections from the PAG and RVM, located in the brainstem, only exhibit adult-like inhibitory properties from P21 (Hathway *et al.*, 2009; van Praag *et al.*, 1991). Hathway and colleagues (2009) showed RVM projections modulate spinal cord activity at all ages; direct electrical microstimulation of the RVM led to facilitation of noxious-evoked spinal reflex activity in rat pups aged P3-P21, whereas in the adult the same intensity of electrical RVM stimulation inhibited noxious-evoked reflex activity. Furthermore, lesions of the RVM in the adult reduced hind-paw mechanical thresholds (due to the lack of regulation) however in the rat pup an increase in mechanical threshold was observed and suggested that an excitatory control had been removed. The tonic influence of RVM projections on the spinal cord undergoes developmental maturation that shifts from faciliatation of spinal reflex activity in the postnatal period to tonic inhibition in the adult (after three weeks of age).

Reflexes present in the neonatal period such as plantar grasp reflex and Babinski reflex disappear in adulthood. Lesions in the supraspinal centres and spinal cord injury lead to the reappearance of reflexes associated with the neonatal period due to the dissociation from descending inhibitory control. The plantar grasp reflex is elicited in healthy infants from 27 weeks GA until 6 months corrected-age and can reappear again in adults with lesions to the nonprimary motor cortex (see Futagi *et al.*, 2010). A positive Babinski's sign is elicited by applying a moving stimulus along the sole of the foot to evoke extension of the toes (toe splaying) in the infant. These characteristics disappear beyond 12-24 months of age and identical stimulation will elicits toe flexion in the healthy adult. Gradual inhibition of reflex activity in the lower limb and the associated disappearance of the plantar grasp reflex and Babinski's sign are indicative of increased inhibition of the developing spinal circuitry.

1.2.2.5 The balance of excitatory and inhibitory neurotransmission

The balance between excitatory and inhibitory neurotransmission is not fully mature in the neonate. The key neurotransmitters acting in the adult spinal cord are glutamate (excitatory), GABA (inhibitory) and glycine (inhibitory); see section 1.1.2.

GABA/glycine:

Synaptic release of GABA activates post-synaptic GABA receptors, and hyperpolarises the cell by allowing the passage of chloride ions from the extracellular to the intracellular compartments. This decreases the neuronal excitability and has a multitude of effects including reduction of nociception. In the very young neonate, GABA and glycine generate an excitatory, rather than inhibitory, effect. $GABA_A$ receptors undergo marked postnatal changes in expression levels, distribution and sub-unit composition (Ma *et al.*, 1993). Application of GABA and glycine induces membrane depolarisation due to a higher intracellular chloride concentration and low expression levels chloride cotransporters (e.g. K^+/Cl^- cotransporter type 2 protein) compared to mature neurons (see Vinay *et al.*, 2000).

Pharmacological studies have been used to understand GABA-mediated inhibitory nociceptive transmission in the spinal cord. Intrathecal application of gabazine, a selective $GABA_A$ receptor antagonist decreases mechanical and thermal thresholds in adults, and increases sensory thresholds in the neonatal rat (Hathway *et al.*, 2006b). Furthermore, Koch and colleagues (Koch *et al.*, 2008) investigated the effect of the benzodiazepine midazolam, a positive allosteric modulator of the $GABA_A$ on sensory thresholds and found a dose-dependent reduction in mechanical and thermal withdrawal thresholds of the hind-limb in young rat pups (P3), this was accompanied with a sensitised EMG response; there was no effect in adult animals. These results indicate that positive allosteric modulation of $GABA_A$ receptors is excitatory in the neonate. Inhibitory networks are important for regulating nociceptive processing. Substantial changes in the organisation and 'tuning' of inhibitory networks occur in the postnatal period. *In vivo* electrophysiological recordings of dorsal horn cell activity show that contralateral inhibitory receptive fields are less spatially restricted than in the adult, and the intensity of inhibitory distribution was more evenly distributed in the neonate (Bremner *et al.*, 2008). These characteristics of inhibition are contributory factors to the enhanced excitability of cutaneous flexion withdrawal reflexes and poorly coordinated responses seen in the neonate that are not present in the adult.

1.2.2.6 Higher brain regions

Extensive cortical development begins postnatally, but little is known of the development of intracortical network connections in infancy. In the human, thalamocortical connections begin to emerge between 23-24 weeks GA (Kostović *et al.*, 2006). Subplate neurons in the cortical plate are transient in nature and an important docking point before many afferent fibres grow into the cortical plate. The subplate recedes after 32 weeks GA whilst the cortical plate matures further. Here, thalamic afferents begin to reach the somatosensory subplate by 20 weeks GA, although neural connections may yet be functional (Kostović *et al.*, 2006). Indeed, many connections between the subplate and cortical plate neurons are important in pain processing and convey information from the thalamus to the developing cortex.

Electroencephalographical measurement of cortical activity is normally asynchronous between hemispheres in young infants and becomes mostly continuous by 36 weeks GA as connections within the cortex mature. Somatosensory evoked potentials following cutaneous stimulation are detectable by 31 weeks GA, indicating that thalamic connections with the somatosensory cortex are functional at this time (Hrbek *et al.*, 1973; Klimach *et al.*, 1988). More recently, changes in cortical haemodynamic activity (as an indicator of neural activity) over the somatosensory cortex are detected in infants as young as 25 weeks GA following a noxious heel lance (Slater *et al.*, 2006). The somatosensory cortex is activated by noxious stimulation from an early age, but little is known of activation in other cortical regions.

1.2.2.7 Summary of developmental characteristics in the rat and human

Human (GA) weeks	Description	Rat (E)mbryonic or (P)ostnatal Days
37-42	Gestational period	**21.5**
9.5	Synapses in ventral horn	**E13.5**
10	Afferent synapses in spinal cord	**E15-19**
10-12	Cutaneous reflexes (perioral to feet/tail)	**E15-19**
15	Lamination in spinal cord	**E17**
23-24	Thalamocortical projections	**P0**
27	Motor neuron death complete	**P0**
29	Somatosensory evoked potential with distinct components	**P10**
32	Decreased excitability of cutaneous reflexes	**P14**

Table 1-2: Comparison of developmental characteristics of the rat and human somatosensory system
Adapted from Fitzgerald (1991) and Lee (2005).

1.3 Pain in the neonatal population

Pain is an unpleasant sensory and emotional experience. Because pain is subjective, the most reliable estimate of pain is through self-report. Adult humans can communicate their pain experience to others and adopt coping strategies to deal with their discomfort, including medical treatment and cognitive-behavioural approaches. Newborn infants are incapable of expressing their pain verbally. The reliance on indirect measures of neonatal pain perception means accurate assessment and appropriately directed pain management remain a major challenge for clinicians.

1.3.1 Pain in the neonatal intensive care unit

The rise in number of preterm births, complemented by advances in technology and medical care, mean that neonates are surviving outside of the womb at much younger gestational ages (Fawke, 2007; Langhoff-Roos *et al.*, 2006; Saigal *et al.*, 2008). An infant admitted to intensive care can expect to receive between 14 - 16 painful or stressful procedures per day (Carbajal *et al.*, 2008; Simons *et al.*, 2003). Commonly performed painful procedures are tissue damaging and include heel lancing and venipuncture, which are required for blood sampling (Carbajal *et al.*, 2008). With an average intensive care stay of 56 days the potential for suffering pain in these infants is clearly significant (Green *et al.*, 2005).

1.3.2 Current clinical pain measurement in neonates

Current measures of neonatal pain are based on behavioural (e.g. change in facial expression, body movements) and physiological (e.g. change in heart rate, blood oxygen saturation) responses. To date over 35 clinical pain assessment tools have been developed for neonates (Duhn *et al.*, 2004). The premature infant pain profile (PIPP) is the best validated (Stevens *et al.*, 1996), and uses facial action including eye squeeze, brow bulge and nasolabial furrow, and physiological measures of heart rate and oxygen saturation, combined with weighting factors such as gestational age and sleep state to give a composite score. These activities can be mediated at the level of the brainstem or below (Fitzgerald, 2005; Fitzgerald, 2009), and do not necessarily reflect activity in higher brain centres.

The immaturity of the neonatal central nervous system means the relationship between noxious stimulation, behavioural and physiological output is difficult to accurately interpret. Sensory reflexes such as the flexion withdrawal reflex can be evoked at lower thresholds

compared to the adult, and may not reflect a noxious-specific response, rather generalised excitability of the spinal cord (Andrews *et al.*, 1999; Fitzgerald *et al.*, 1988b). Heart rate increases rapidly in response to nociceptive input; this physiological measure is highly variable and non-specific to pain reactivity, and additionally limited by causal effects of underlying illness (Oberlander *et al.*, 2002; Stevens *et al.*, 1995). Given the requirement of cortical involvement for pain experience (Tracey *et al.*, 2007), surrogate measures of pain may not provide a complete representation of the neonate's experience. Slater *et al* (2008) have showed changes in cortical haemodynamic activity are well correlated with clinical pain scores, suggesting that painful stimulation generally evokes parallel cortical, behavioural and physiological responses. In some infants, however, cortical activity could still be measured without a concurrent behavioural change, which implies that pain assessment based on behavioural tools alone may not accurately reflect the infant pain experience.

1.3.3 Management of neonatal pain

Treatment of neonatal pain is challenging for clinicians. Procedural pain treatment often consists of a multimodal approach using a combination of pharmacological and non-pharmacological methods. Despite awareness that neonates are exposed to many potentially painful procedures, clinicians are hesitant to administer analgesia. Indeed there is huge variation in clinical practice regarding the administration of specific, pharmacological analgesia for painful procedures in neonates. For instance, one study reported in 8 neonatal intensive care units administration of specific pain relief ranged from 5 – 50 % (Carbajal *et al.*, 2008). Clinicians are rightly concerned about the efficacy of pharmacological therapies and the associated adverse effects of the analgesia. Opioids, for example, can be administered to alleviate severe pain but adverse effects include respiratory depression, hypotension, muscle rigidity and increased risk of necrotising enterocolitis (Simons *et al.*, 2003). In addition, the effectiveness of pain relief has also shown to be procedure-specific. Topical anaesthesia using EMLA 5% cream (eutectic mixture of local anaesthetics, lidocaine and prilocaine) is more effective against venepuncture than heel lance, probably due to differences in the depth of stimulus penetration (Taddio *et al.*, 1998). Sucrose decreased clinical pain scores following venepuncture but was ineffective during intramuscular injections or heel lance (Taddio *et al.*, 2008). Non-pharmacological approaches such as cuddling, swaddling and suckling can reduce behavioural and physiological responses to pain and are implemented in many neonatal units but are not considered an adequate substitute for analgesia (Howard, 2003; Johnston *et al.*, 2010).

The developing central nervous system is not simply scaled down from the adult. Nociceptive circuits are subject to refinement and fine-tuning after birth. Age-related changes in pharmacokinetic variables occur including organ maturation e.g. renal function; total body water and changes in the drug elimination pathways (Walker, 2008). Developmentally regulated changes in receptor expression and distribution e.g. opioid and glutamate receptors, and neurotransmitter function influence the pharmacodynamic profile to modulate the analgesic effectiveness and toxicity (Fitzgerald *et al.*, 2009; Walker, 2008). If pain relief is to be routinely administered for neonates, clinicians need evidence of its analgesic efficacy. The paucity of agents tested specifically on this vulnerable group increases the difficulty in making an informed judgement on the risk-benefit balance of providing analgesia.

1.3.4 Long term effects of tissue injury in the neonatal period

Evidence strongly suggests that injury in early life can lead to long-term alterations in pain processing. The immediate effects of repeated heel lancing result in heightened behavioural responses that can last hours or days and is attributable to local peripheral changes around the site of injury (Abdulkader *et al.*, 2008a; Fitzgerald *et al.*, 1988a; Taddio *et al.*, 2002). In agreement with these data, abdominal reflex studies show mechanical hypersensitivity in infants who underwent abdominal surgery compared to healthy controls (Andrews *et al.*, 2002a; Andrews *et al.*, 2002b).

Prolonged effects of neonatal tissue injury also exist beyond the neonatal period. Infants born prematurely experience a higher incidence of invasive procedures as part of their essential medical care and exhibit sustained hypersensitivity to innocuous mechanical stimuli, as measured by flexion withdrawal reflex activity, during the first year of life (Abdulkader *et al.*, 2008b). Recent work by Slater and coworkers demonstrate that premature infants hospitalised for at least 40 days exhibit increased cortical responses to noxious stimuli compared to healthy term infants. Further, neonatal circumcision is frequently performed within the first 5 days of life, circumcised infants exhibit heightened behavioural responses to routine vaccination at 4-6 months of age compared to uncircumcised infants; this was partly attenuated with topical local anaesthetic (EMLA cream) application prior to the initial surgery (Taddio *et al.*, 1997). These effects may outlast the period of injury into childhood.

Longitudinal studies on the neurological development of infants born prematurely or hospitalised in the neonatal intensive care unit have used quantitative sensory testing (QST) to examine sensory function at school age. QST provides objective and quantifiable measures of

sensory thresholds of different modalities in children (Blankenburg *et al.*, 2010). Preadolescent children previously admitted to neonatal intensive care exhibit increased heat sensitisation and lower basal sensory thresholds when compared to age-matched controls (Hermann *et al.*, 2006; Walker *et al.*, 2009a). School-age children of the similar age, who underwent cardiovascular surgery during the neonatal period, exhibit altered somatosensory perception when stimulated around the region of tissue-injury (Schmelzle-Lubiecki *et al.*, 2007). Additionally, work supporting these findings by Walker et al (2009a) found that alterations in thermal perception were more significant in former extremely-premature children who underwent surgery during the neonatal period. Global changes in thermal and mechanical sensitivities are proposed to be due to centrally-mediated alterations in nociceptive pathways (Walker *et al.*, 2009a). Collectively these data show that injury in early life can change baseline sensory function and enhance responses to future pain

Non-invasive tools such as electroencephalography (EEG) and functional MRI are being utilised with increasing frequency to further our knowledge of the neural circuitry underlying sensory processing in higher regions of CNS. Whilst much neuroimaging research has been widely applied to investigate pain perception in the adult, relatively few comparable studies have been undertaken in children. Recent work by Hermann and coworkers, used functional MRI to investigate regions of brain activity during sensory processing in children (aged 11-16yrs) who were former neonatal intensive care patients. Children born prematurely exhibited increased haemodynamic activity in the primary somatosensory cortex, anterior cingulate cortex and anterior insular compared to full-term during thermal heat stimulation at moderate pain intensity (Hohmeister *et al.*, 2010).

Activity-related plasticity from neonatal pain experience(s) may contribute to the reorganisation and strengthening of neuronal networks in later life. It has been demonstrated in the rat that the normal postnatal maturation of dorsal horn sensory circuits critically depends upon the correct pattern of afferent sensory input (Waldenstrom *et al.*, 2003) and upon NMDA-dependent synaptic activity in the spinal cord (Beggs *et al.*, 2002; Pattinson *et al.*, 2006). Neonatal nociceptive circuitry is evidently especially sensitive to early tissue injury and pain. Excitatory postsynaptic synaptic currents (EPSCs) in lamina II of the dorsal horn are increased following skin injury in at P3 or P10, but not at P17 (Li *et al.*, 2009) and skin incision in the first postnatal week, but not later, increases hyperalgesia following repeat surgery two weeks later (Walker *et al.*, 2009b). It is still not clear whether these cellular and

synaptic changes are the underlying mechanisms for the prolonged effects of neonatal tissue injury observed in children.

1.4 Aims of the thesis

It is clear from animal studies that nociceptive processing in the spinal cord is subject to fine-tuning throughout the postnatal period. Immature spinal sensory reflexes have lower mechanical thresholds and are poorly coordinated and exaggerated compared to adult reflexes. However, little quantitative data is available on how these spinal sensory circuits develop and are modulated in the human infant. This thesis investigates the development of cutaneous flexion withdrawal reflexes in preterm and full-term human infants using surface EMG recordings of the lower limbs. Three main aspects of neonatal sensory processing were the focus of this research.

In Chapter 3 (Study 1), the properties of the flexion withdrawal reflex are comprehensively characterised following noxious and non-noxious mechanical stimulation, with the aim of understanding the selective effects of noxious procedures upon spinal circuits in preterm and term infants. The work focuses on the development of the response at preterm and full-term gestational age, stimulus specificity, motor reflex co-ordination of the lower limbs and the relationship with observed facial behaviour.

In Chapter 4 (Study 2), cutaneous sensitivity and plasticity of the spinal cord to mechanical stimulation was investigated, with the aim of understanding the effects of repeated handling and cutaneous mechanical disturbance upon spinal circuits in preterm and term infants. The study characterises the development of sensory flexion reflex thresholds to single mechanical stimuli using calibrated forces and the associated flexion withdrawal reflex activity. In addition the effect of a short series of repeated stimuli on changes in sensory thresholds and reflex activity are examined.

In Chapter 5 (Study 3), the modulation of cutaneous flexion withdrawal reflex activity to oral sucrose was investigated, in a randomised controlled trial, with the aim of understanding the effects of a commonly administered analgesic upon spinal circuits in preterm and term infants. The analysis techniques optimised in Study 1 were used as a basis for data analysis. The effect of oral sucrose versus sterile water on flexion withdrawal reflex activity following a clinically required heel-lance was tested in healthy full-term infants, and compared to clinical pain assessment scores.

Chapter 2

General Methods

2 General Methods

2.1 Research with human subjects

All of the outlined studies were conducted on in-patients admitted to the Elizabeth Anderson and Obstetrics Wing, University College Hospital (UCH). When working with human subjects, particularly newborn infants, it is essential to (1) have ethical approval, (2) adhere to the rules and protocols of the host institution, (3) recruit a suitable study cohort, and (4) maintain an appropriate level of data protection throughout the research process.

(1) Ethical approval

Ethical approval was obtained from the UCH research ethics committee and informed written parental consent was obtained prior to each study. The study conformed to the standards set by the *Declaration of Helsinki.*

(2) Conducting neonatal research at UCH

All clinical research performed on the neonatal unit required parental consent, and gave careful consideration to both infant wellbeing, and health and safety. Studies were carefully scheduled to minimise subject discomfort and avoid concurrence with potentially confounding clinical treatments or procedures.

Authorisation from the parent(s) (or legal guardian) was obtained before an infant was studied. When recruiting an infant for clinical research it was important to recognise that some parents were likely to be experiencing emotional stress as a result of their child's hospital admission and to adopt a sympathetic approach.

The infant's wellbeing was always a priority for the research team; experiments were designed to minimise risk or discomfort to the infant. A research nurse was present throughout each study to monitor clinical stability and perform the heel lance procedure (where applicable). Emergency equipment and trained medical personal were available to deal with the unlikely event of any harm or adverse effect to the infant. All studies were conducted at the cot-side. Physiological recording equipment was compatible with the hospital

equipment. All electronic devices were safety tested by the UCH health and safety team before using on the neonatal unit.

Prior to entering a nursery or ward, or to patient handling, hands were washed thoroughly to reduce the spread of infection in adherence with UCH's strict infection control regime. Latex gloves and plastic aprons were worn to provide an additional level of protection to the patient and researcher during each study. Cross-infection was reduced by identifying infants with infection and excluding them from the study. Aseptic techniques were used throughout all studies; all surface electrodes and heel lancet devices were sterile, and discarded after use. After each experiment, all recording equipment was disinfected with alcohol cleansing wipes.

Research was undertaken only when a clinical blood sample was required and where necessary, the clinical procedure was performed without research being undertaken. The time chosen to conduct a study was coordinated around (1) the necessity of the blood test results, (2) clinical care i.e. infants subjected to invasive procedures such eye-examinations for retinopathy of prematurity were not studied on the same day, and (3) the capability of the infant to tolerate additional handling.

(3) Infant recruitment

Infants suitable for inclusion in the study were identified by the research nurse and neonatal consultant. Parents were asked whether they would consider their child to be involved; the rationale for the study and procedure were discussed to ensure the parents were fully informed of the research process and had the opportunity to ask questions. Supplementary written material was also given. Participation was entirely voluntary. There was no time limit for parental consent. Infants were recruited to the study if their parents agreed to sign a consent form. Once enrolled onto the study parents were free to withdraw permission at any time.

UCH has between 3000-4000 deliveries each year, with 400-500 being admitted to the neonatal intensive care unit (NICU). Approximately 10 % of annual NICU admissions are extremely premature infants, (born at less than 28 weeks gestation) and under half of these are stable enough cope with additional handling involved in the study.

The time taken to identify an infant suitable for inclusion in the study, approach the parents and conduct the experiment was typically approximately 6hrs, ranging between 3hrs and several days. The clinical stability of the infant and acquisition of parental consent were factors that prolonged this time period.

(4) Data protection

All personal information was held in accordance with the Data Protection Act (1998) and was registered with the *UCL Data Protection Register*. Allocation of a unique four-digit identification number for individual infants and each test occasion was conducted to ensure patient anonymity before data was recorded on the UCL laboratory electronic and paper data storage systems.

For each participant, demographic data, clinical history and clinical status at the time of study were logged in a database. Data files were password protected and paper records stored in a locked filing cabinet in a secure room at UCL. Only those researchers with an honorary or a full-time contract to work at UCH had access to the participant's details and their hospital records.

2.2 Participants

Prior to each study the clinicians in the research group assessed wellbeing and suitability of the infant for the research. Studies were postponed or cancelled where the infant was considered to be unfit to take part.

Infants were not eligible for inclusion in a study if they (1) showed signs of tissue damage on the lower limbs, (2) had intraventricular haemorrhage or periventricular leukomalacia, (3) were receiving analgesics or sedatives, (4) were born to mothers who were opioid users, or (5) were born with known congenital malformations or genetic conditions.

2.3 Study design

This thesis comprises three related studies. For simplicity, an overview of each study is given below and described in more detail in 'Methods' section of each study chapter.

Study 1: Characterisation of nociceptive and non-nociceptive flexion withdrawal reflex activity

A clinically required noxious heel lance and non-noxious touch stimulus were performed on the heel at the same site in each infant. Spinal flexion reflex withdrawal activity and facial behaviour were measured using surface EMG and video recording techniques respectively.

Study 2: Cutaneous sensory thresholds and flexion withdrawal reflex activity following single and repeated mechanical stimulation

Cutaneous sensory threshold, and the effect of repetitive stimulation at suprathreshold intensities and the subsequent cutaneous sensory threshold were investigated. Mechanical stimulation of the plantar surface of the foot was conducted using a series of calibrated von Frey hairs. Spinal flexion reflex withdrawal activity was assessed using surface EMG recordings.

Study 3: Oral sucrose as a modulator of nociceptive spinal flexion reflex withdrawal activity[1]

A randomised controlled clinical trial was conducted to investigate the effectiveness of oral sucrose as a modulator of spinal nociceptive processing in healthy infants. Subjects received 0.5ml of a solution of 24% sucrose or sterile water onto the tongue, two minutes prior to a clinically required heel lance procedure. Spinal flexion reflex withdrawal activity was measured using surface EMG recordings. Clinical pain assessment using behavioural observations and pulse oximetry was also conducted.

[1] Slater, R, Cornelissen, L, Fabrizi, L, Patten, D, Yoxen, J, Worley, A, Boyd, S, Meek, J, Fitzgerald, M (2010a) Oral sucrose as an analgesic drug for procedural pain in newborn infants: a randomised controlled trial. *Lancet* **376**(9748)**:** 1225-1232.

2.4 Stimulus

In Study 1, a noxious and non-noxious touch stimulus was applied to the heel using a lancet device. For the purposes of Study 3, only a noxious heel lancet was applied to the foot.

2.4.1 Noxious stimulus

Clinical heel lance procedure

The noxious stimulus was a clinically required heel lance. This was a routine procedure for blood sampling performed by a trained clinician using a lancet device (Figure 2-1). The lancet device was held against the outer aspect of the heel, a spring-loaded blade was released from the device to break the surface of the skin and expose the capillary bed. For safety reasons, the blade was automatically retracted into the device to avoid injury to the clinician or patient.

The heel lance procedure was conducted when the infant was clinically stable; determined by a period of steady oxygen saturation level (±2%), heart rate, and EMG activity lasting at least 10 seconds. The heel was exposed, cleaned with a sterile swab, and held gently by the clinician throughout the procedure.

Following the heel lance procedure, the foot was not squeezed for a period of at least 30 seconds to ensure that the recorded response could be timed from a single event. This was conducted without impairing the clinical blood collection. No heel lances were performed solely for the purpose of the study.

The lancet device was standardised to ensure consistent incision depth and length in each clinical blood sampling procedure. There were two types of lancet device available for infant blood sampling; each had the same mechanism of action but differed in the size of incision made. Lancet 1 was used for infants weighing <2.5kg, the skin incision size was 0.85mm depth x 1.75mm length; Lancet 2 was used for infants weighing ≥2.5kg and made a larger incision of 1.00mm depth x 2.50mm length.

2.4.2 Non-noxious touch stimulus

The non-noxious touch stimulus device was a lancet identical in size to the noxious stimulus lancet used in each study. The lancet device was rotated by 90º and placed against the heel, so that when the spring-loaded blade was released it did not contact the heel and break the skin

(Figure 2-1). Infants experienced the tactile sensation and auditory click that was associated with blade release from the lancet device but no skin incision.

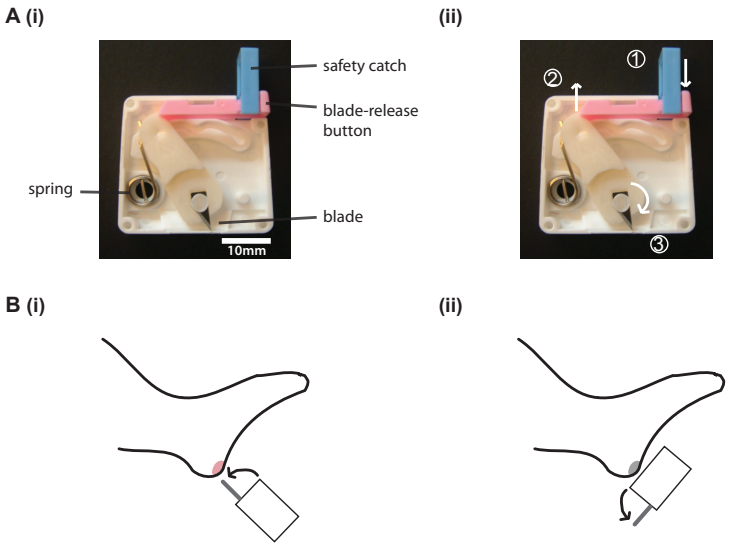


Figure 2-1: Application of noxious and non-noxious stimulus
(A) Cross section of actual lancet device (i) Key features are labelled; (ii) Mechanism of blade release: (1) The blade-release button is pressed downwards, (2) button lever pivots upwards to release tension on the spring, (3) blade is released and rotates around until is rapidly drawn back into the device by the spring.
(B) Position of lancet device for each modality (i) Noxious stimulus: lancet device is positioned with blade release site against the heel; as the blade is released the skin is incised. (ii) Non-noxious stimulus: lancet device is positioned against the skin but with blade release facing away from the heel, there is no contact with the skin during blade release. Shaded regions illustrate the approximate region of skin in contact with the lancet device. Arrows indicate direction of blade movement. Not to scale.

2.4.3 Von Frey hair stimulus

For Study 2, a series of calibrated von Frey hairs were used to apply a range of forces for sensory testing. Von Frey hairs are monofilaments made of nylon and designed to exert a precise force when applied perpendicularly to a surface. As illustrated in Figure 2-2, precision is facilitated because the monofilament is applied with increasing force until it bends and the force exerted onto the surface can no longer increase (Bell-Krotoski *et al.*, 1987). The size of the force applied is dependent on the width of the nylon monofilament and arranged logarithmically in a series of monofilaments, from lightest to heaviest (Figure 2-3). In this study, von Frey hairs were applied to the same site on the medial-plantar surface of the foot. The conversion from von Frey hair number to grams for the set used is listed in Table 2-1.

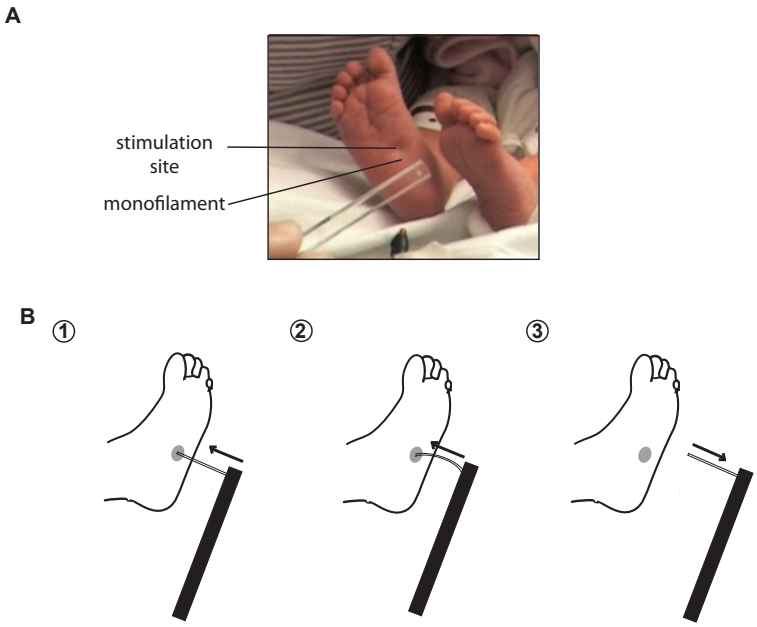


Figure 2-2: Application of von Frey hairs
(A) Actual von Frey hair application in a full-term infant (GA 38.86 weeks). Skin indentation is observed as pressure is applied to the foot. (B) Method of von Frey hair application: (1) Initial application of monofilament at 90° to the medial-plantar surface of the foot (2) Bending of the monofilament to exert maximum force for 3 seconds (see photo in (A) also). (3) Removal of monofilament. Arrows indicate direction of direction of force applied. Not to scale.

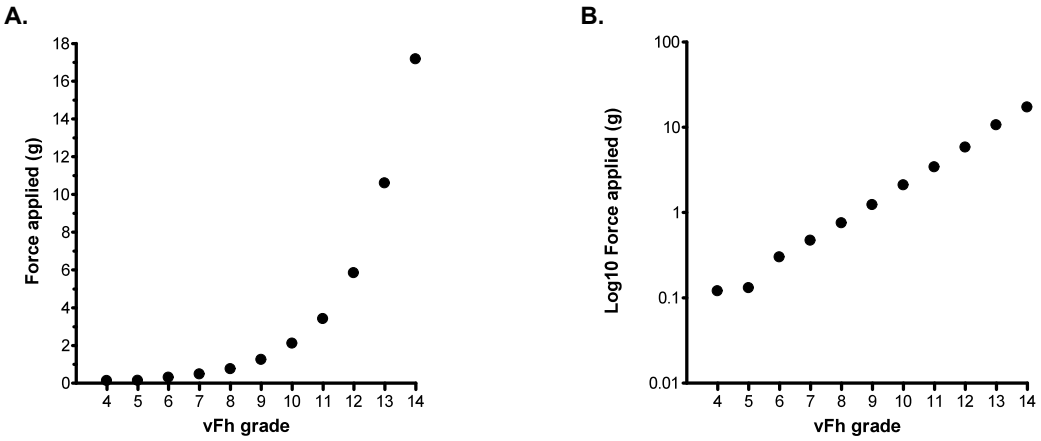


Figure 2-3: Calibration of von Frey hairs used
Actual forces applied: (A) Linear interval and (B) Logarithmic interval between grades of von Frey hairs.

Von Frey hair number	Force (g)
4	0.12
5	0.13
6	0.30
7	0.47
8	0.75
9	1.23
10	2.10
11	3.40
12	5.83
13	10.60
14	17.17

Table 2-1: Actual forces of von Frey hairs used

2.5 Event-marking

2.5.1 Noxious and non-noxious touch stimulus

The time of noxious and non-noxious touch stimuli applications were automatically event-marked by electronically linking the lancet device to the recording equipment. An accelerometer connected to the recording equipment was used to sense the movement of the spring-loaded blade from the lancet when triggered by the clinician. The accelerometer was physically attached to the heel lancet device using a self-adhesive fixative. Blade release from the lancet displaced the accelerometer and triggered an event-mark on the recording within a latency of 1ms (Worley *et al.*, 2006).

Where video recordings were conducted for analysis of facial behaviour (Study 1 and Study 3), the displacement of the accelerometer simultaneously activated a light-emitting diode (LED) to flash and visually mark the video footage as well as the electrophysiological recording.

2.5.2 Mechanical stimulation

Electronic von Frey hair kits have been designed to record the latency and force applied for limb withdrawal. Such technology has not been developed with sensitivity to forces less than

1g and was therefore unsuitable for sensory threshold testing in the human infant for Study 2 (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). In collaboration with Mr Alan Worley, preliminary work in this PhD attempted to develop an automatic event-marking system for the application of forces less than 1g. This event-marking system was capable of detecting monofilament movement at low forces i.e. <1g, but lacked stimulus specificity; false event-marking would occur when the handle of the device was moved through the air, in the absence of contact between the monofilament on surface of interest.

Development of an automatic event-marking system for use with von Frey hairs was beyond the scope of this thesis. Therefore, von Frey hair application was manually event-marked using a custom-built push-button trigger system. The push-button was a hand-held device and was linked electronically to the recording equipment to mark the recording once the button was manually pushed downwards. The researcher applied the mechanical stimulus with one hand and pressed a push-button with the free hand as close to the point in time where the monofilament contacted the skin. The researcher was trained in using the push-button system prior to actual data acquisition to ensure reproducibility of event marks. To test the latency error, a pilot study was performed by simultaneously videoing stimulus application with the manual event marking. The event was manually marked within one video frame: 20ms. Where the push-button was accidently pressed the researcher manually triggered the button several times to indicate on the recording a false event-mark.

2.6 Data acquisition

2.6.1 Electromyography (EMG) recording

Motor activity was recorded from the biceps femoris muscle using self-adhesive bipolar surface silver/silver-chloride (Ag/AgCl) electrodes (Cardinal Health, Germany). The skin was cleaned and lightly abraded with an EEG prepping gel to reduce electrode/skin impedance. Pairs of electrodes were positioned over the muscle as far apart as possible depending on gestation i.e. 30weeks GA: 25-30mm, 43weeks GA: 35-40mm; one pair per muscle. Electrodes were secured in place using a self-adherent wrap around the lower limb and electrode leads were tied together to minimise external electrical influence.

The EMG signal was amplified (10,000 times), filtered between 0-500 Hz and sampled at 2 kHz using a high-resolution recording system (Neuroscan Synamps 2, Compumedics, USA). A chest electrode served to ground the signal. A notch filter was applied at 50Hz to reduce

interference from external electrical signals. Data files were recorded using Scan 4.3 Neuroscan software (Compumedics, USA). Figure 2-4 illustrates the major components of the recording system.

2.6.2 Video recording

Video footage was obtained using a camcorder (JCV, Everio hard disk camcorder). A moveable LED was positioned in the field of view and flashed to indicate the time of stimulus on the video. For Study 1 and Study 3 the camera field of view was positioned such that the whole face was recorded for facial expression analysis. In Study 2, the field of view required was the stimulus site and lower limbs in order that visual inspection could be used to ensure that intra-stimuli applications were located at the same site on the foot.

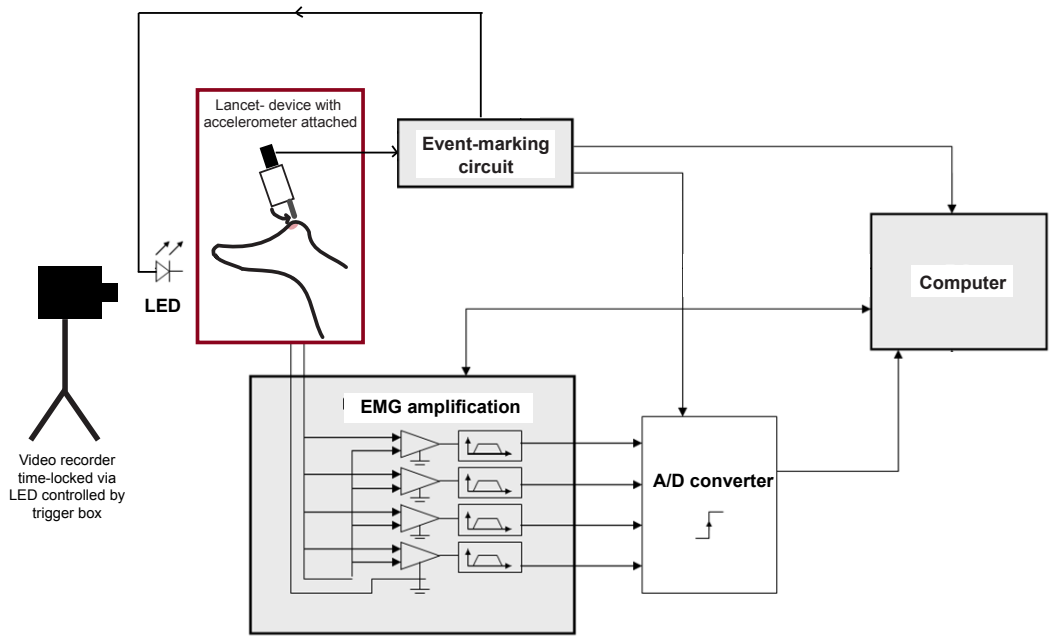


Figure 2-4: Basic components of the automatic event-marking data recording system
Adapted from Worley et al (2006). A/D converter: analogue to digital signal converter; EMG: electromyography; LED: light-emitting diode.

2.7 Data analysis

2.7.1 EMG analysis

Raw EMG recordings were epoched, filtered and rectified using Scan 4.3 Neuroscan software (Compumedics, USA). Epochs of the raw EMG recordings were made 1000ms before and 5000ms after the stimulus. The recordings were filtered with a 10Hz high-pass filter to eliminate signal drift and ensure signal composition contained frequencies associated with motor activity (Figure 2-5). For latency detection the EMG signal was converted from a mix of positive and negative polarity into positive-only values by full-wave rectification. Files were exported to customised MatLab software (MathWorks, USA) for data analysis.

EMG recordings were not eligible for inclusion in the final analysis if technical failure or movement in baseline period occurred. Technical failure included (1) no event-mark, (2) 50Hz activity, or (3) no EMG signal (0mV)/ gross activity (>265μV) due to poor electrode-to-skin contact. Movement in the baseline period was defined as EMG activity that exceeded 35μV between -1000ms and the time of stimulus.

Pilot analysis was performed on EMG recordings acquired from a set of full-term infants after a noxious heel-lance stimulus (n=19). The purpose was to clearly define flexion withdrawal reflex activity and optimise methods to quantify EMG activity. A clear reflex response to heel stimulation was defined as a change in EMG activity that exceeded 3 standard deviations (SD) of baseline activity (Figure 2-6). Standard deviation was used as a measure of signal variance and thus to detect a change in EMG activity following the stimulus because baseline activity was stable for all infants. A series of voltage thresholds ranging from 1 – 4 SD above baseline activity were tested for onset of reflex response detection and compared against visual inspection. The optimal voltage threshold was 3SD above baseline because the onset latency values calculated were most comparable to visual inspection values (Appendix I). The magnitude of EMG activity was quantified by dividing each epoch into 250ms time bins and calculating the root mean square (RMS) for each time bin over the -1000 to 5000ms recording period (Figure 2-6-B). A selection of time periods ranging between 50ms to 500ms was tested on the pilot data to find the most suitable time-bin. The 250ms time bin was chosen because smoothing of the EMG signal was permitted without masking gross changes in the pattern of activity.

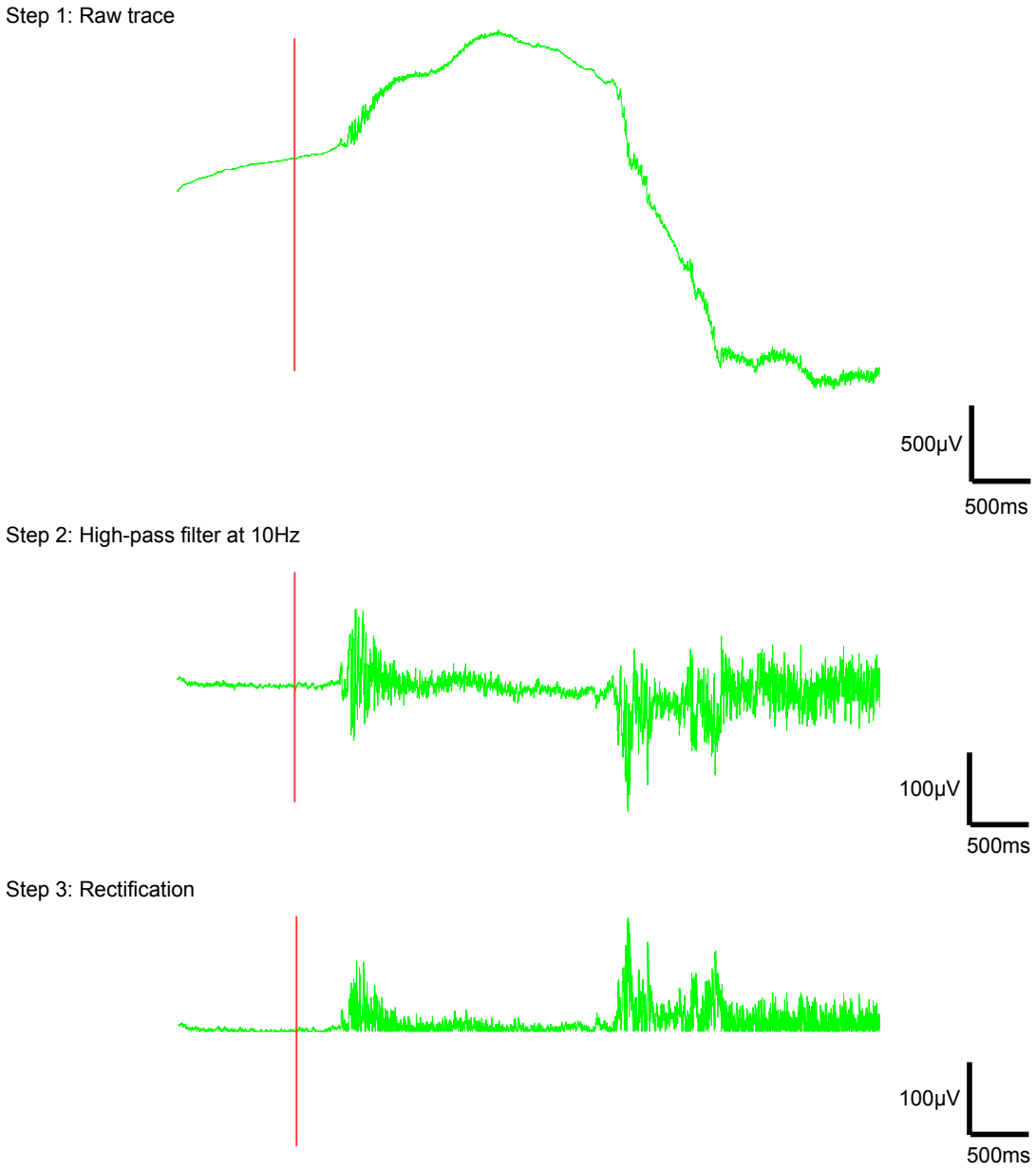


Figure 2-5: Steps used to transform the raw EMG recording for quantification
Example EMG recording from a full-term infant at GA 39.43 weeks. Step 1: Raw EMG trace acquired; Step 2: Raw EMG trace is high-pass filtered at 10Hz; Step 3: Filtered trace is full-wave rectified. Red line indicates time of noxious heel lance. Scale bars are on the right-hand side of each trace.

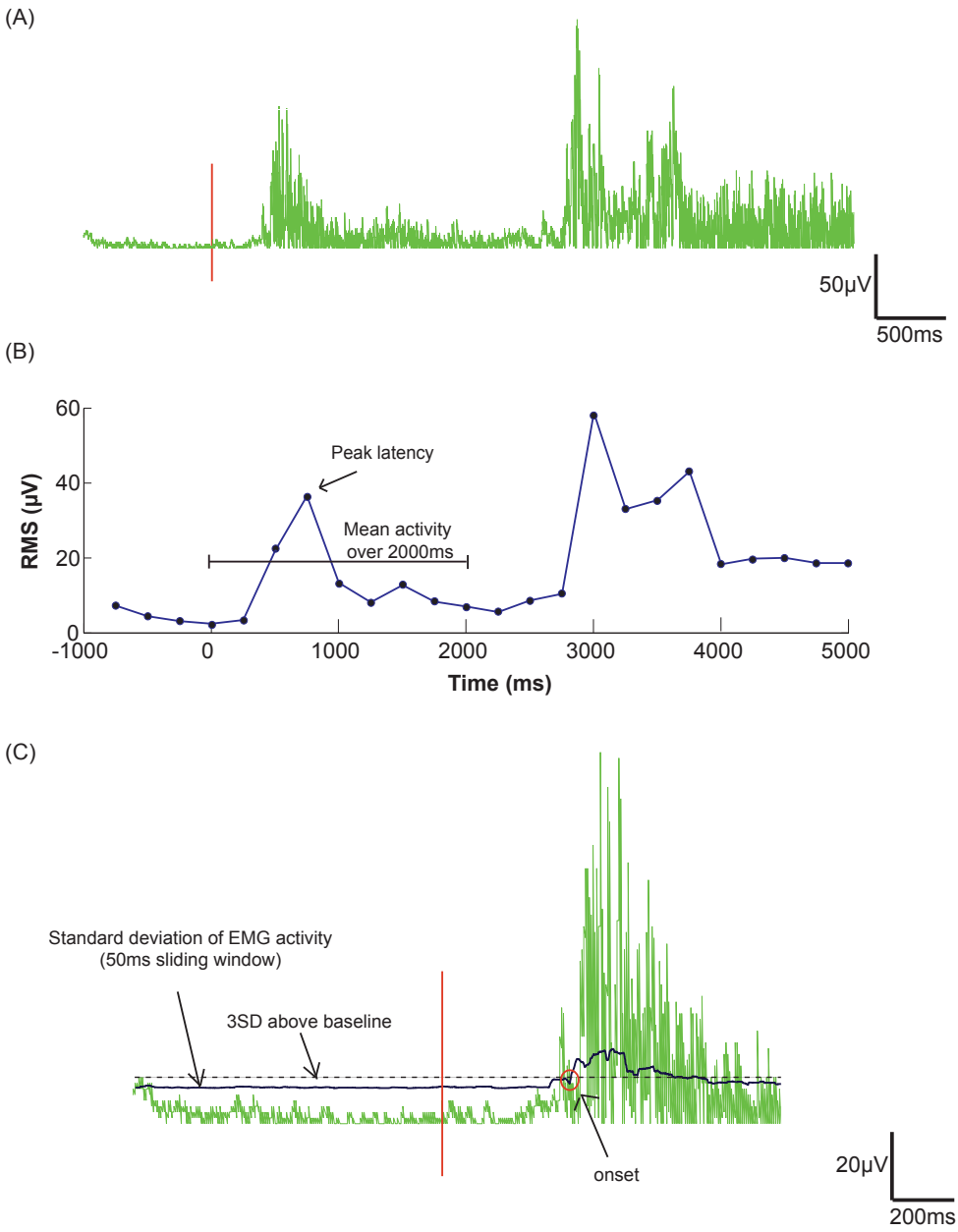


Figure 2-6: Measures used to characterise flexion withdrawal reflex activity
Example EMG recording from a full-term infant at 39.43 weeks GA following a noxious heel lance. (A) EMG recording (post-filtering and rectification) shows clear flexion withdrawal reflex activity; (B) EMG recording is separated into 250ms time-bins and the RMS calculated for each time-bin. Mean (RMS) activity over 2000ms and peak latency are also determined. Stimulus is applied at time 0. (C) Onset latency detection; blue line, the standard deviation over sequential 50ms time-window; horizontal dashed line, 3SD above baseline (threshold); red circle, where EMG activity exceeds the threshold. Onset latency for this infant is 408.5ms. Red line indicates time of noxious heel lance. Scale bars for each trace are on the right-hand side of the figure.

The following set of analyses were used to characterise the reflex response:

(1) Latency

Onset latency: the time (ms) between the stimulus and the onset of evoked EMG activity (Figure 2-6-C).

Peak latency: the time (ms) between the stimulus and the time-bin where peak EMG activity occurs (Figure 2-6-B).

(2) The pattern of activity

Non-latency corrected analysis: The root mean square of EMG activity (μV) measured in 250ms time bins from 1000ms before (-1000ms) to 5000ms after (5000ms) the stimulus [Figure 2-6-B].

Latency-corrected analysis: The same EMG recordings were also analysed using a method that corrects for variability in onset latency. The recordings were aligned from the time of onset of activity (see point 1 'Latency' above) and analysed in 250ms time bins as above.

(3) Mean amplitude

The root mean square of EMG activity (μV) measured in a single 2000ms time-bin from the time of stimulus to 2000ms after the stimulus.

2.7.2 Facial behaviour analysis

Facial behaviour is used as an indicator for clinical pain assessment in non-verbal patients. In Study 1 and Study 3 the observed changes in facial expression were characterised using behavioural elements of the 'Premature Infant Pain Profile' (PIPP) clinical pain assessment tool. PIPP is a validated clinical pain assessment tool that integrates behaviour, age and physiological parameters to give a numerical score (Ballantyne *et al.*, 1999; Stevens *et al.*, 1996). Here, facial behaviour was scored in the baseline period (15s before the stimulus) and over the 30s period after the stimulus (Table 2-2). Infants with facial behaviour in the baseline periods were excluded from the analysis. A facial response was defined as the presence of either a brow bulge, eye-squeeze or nasolabial furrow in the post-stimulus period. Videos were epoched at 15s before and 30s after the stimulus, as identified by a LED flash event-marking on the video recording, using video editing software (Cyberlink Power Director Express, CyberLink Corp., Taiwan).

The following parameters were used to measure facial behaviour after the stimulus:

(1) Incidence:

The number of infants expressing a visible facial response (n/N; %)

(2) Latency:

The time (s) between stimulus and the first visible facial response

(3) Total facial score (TFS):

The duration of brow bulge, eye-squeeze and nasolabial furrow facial features were individually scored and combined to give a total score (Table 2-2)

	SCORE			
Indicators	**0**	**1**	**2**	**3**
(1) Age (weeks)	x ≥ 36	32 ≤ x <36	28 ≤ x < 32	x < 28
Baseline characteristics				
(15 seconds pre-stimulus)				
(2) Behavioural score	Eyes open; Facial movement present	Eyes open; No facial movement	Eyes closed; Facial movement present	Eyes closed; No facial movement
Post-stimulus characteristics				
(30 seconds post-stimulus)				
Physiological score				
(3) Maximum change in heart rate (bpm)	0 – 4	5 – 14	15 – 24	≥ 25
(4) Minimum change in oxygen saturation (%)	0 - 2.4	2.5 – 4.9	5.0 – 7.4	≥ 7.5
Facial score (% observation time)				
(5) Brow bulge	x <10	10 ≤ x > 39	40 ≤ x < 70	x ≥ 70
(6) Eye squeeze	x <10	10 ≤ x > 39	40 ≤ x < 70	x ≥ 70
(7) Nasolabial furrow	x <10	10 ≤ x > 39	40 ≤ x < 70	x ≥ 70

Table 2-2: Premature Infant Pain Profile (PIPP) clinical pain assessment tool
The PIPP score a composite pain assessment scale made of 7 key indicators. . Behavioural components of the PIPP encompass (2) and (5-7); physiological components of the PIPP include (3) and (4). Facial expression components ('baseline characteristics' and 'facial score') used in facial behaviour analysis are outlined in blue. Bpm. Beats per minute.

2.8 Statistical analysis

Statistical analyses were conducted in Microsoft Excel (Microsoft, USA) and Graph Pad Prism (GraphPad Software, USA). All data in the text and tables is expressed as mean (95% Confidence Interval (CI) limits), unless otherwise stated. Graph error bars are expressed as standard error of the mean (SEM). Detailed statistical analysis has been described in the relevant Methods section for each Study. Significant differences were assumed at $p<0.05$. All groups of data were tested for normality using the D'Aguostino-Pearson normality test, where the data did not follow a Gaussian distribution an appropriate non-parametric test was applied (specified in the text parenthesis).

Chapter 3

Study 1

Characterisation of nociceptive and non-nociceptive flexion withdrawal reflex activity in the human infant

3 Study 1

3.1 Introduction

Premature infants undergo numerous painful and stressful procedures each day as part of their essential medical care; heel lancing for blood collection is one of the most common painful procedures performed (Carbajal *et al.*, 2008; Simons *et al.*, 2003). The importance of understanding the effect of nociceptive and tactile input on the developing CNS is imperative for assessing adverse consequences and for improving clinical treatment (Fitzgerald *et al.*, 2009).

Spinal flexion withdrawal reflex activity is a useful measure of CNS sensitivity to cutaneous sensory inputs in the adult human and animal (Schouenborg *et al.*, 1992; Weng *et al.*, 1996; Woolf, 1984). Activity in the developing spinal cord can be measured with recordings of flexor muscle activity, using surface EMG, in response to mechanical stimulation of the heel and will contribute to our understanding of sensory processing in the neonate (Andrews *et al.*, 1999; Andrews *et al.*, 2000).

3.1.1 Spinal flexion reflexes in the adult

The flexion withdrawal reflex is the simplest centrally organised response to noxious stimuli. Primary afferent fibres carry noxious information from the periphery to the dorsal horn for integration via a network of spinal interneurons, projection neurons and motor neurons, and are regulated by descending input from higher brain regions. Reflexes are adapted to provide the most appropriate movement of the limb away from the stimulus. Sherrington (1910) originally described classic flexion reflex behaviour as movement of the ipsilateral limb towards the body accompanied by a contralateral limb extension; this was based on studies of electrically stimulated withdrawal of the hind limb in decerebrate spinalised cats. He stated that the reflex was most easily elicited from the foot, had a receptive field that encompassed the entire limb, and was readily evoked at noxious levels of intensity. Over the last 60 years a number of detailed investigations have progressed from Sherrington's initial studies of flexion withdrawal reflex activity in the cat to that of the human; it is well understood that flexion reflex activity is similar to that of other vertebrates.

The transmission of sensory information afferent components of the human spinal reflex arc is mediated by noxious-specific A∂- and C-fibres, and carry high-intensity information to the dorsal horn of the spinal cord as shown by human motor reflex studies (Dimitrijević *et al.*, 1970; Ertekin *et al.*, 1975; Hugon, 1973; Kugelberg, 1948). Efferent output, the pattern of flexion motor activity, consists of a 'double-burst', characterised by an initial (RII) tactile component of short latency and low threshold, and a late (RIII) component of longer latency and noxious-specific (Hugon, 1973). The electrical threshold of RII is significantly below the pain threshold (Ellrich *et al.*, 1998; Sandrini *et al.*, 1986). However, it must be noted that the RII innocuous-associated component, may or may not be observed, and is not seen in all subjects (Hugon, 1973; Willer, 1977). Meanwhile, the RIII component is always present and is related to actual limb withdrawal.

Noxious stimulation of various skin locations evokes coordinated flexion withdrawal movements, of which most of the muscles in the lower limb participate. Reflex patterns of movement are functionally organised to ensure optimal withdrawal in relation to the site of stimulation, and are designed primarily to defend the limb whilst simultaneously maintaining posture. For instance, stimulation of the sole of the foot in man evokes a general flexion-reflex in the ipsilateral leg accompanied with a stiffening of the contralateral leg (Kugelberg *et al.*, 1960). Hagbarth characterised reflex behaviour in a number of extensor and flexor muscles relative to the stimulation site, and showed the reflex was a well-directed movement, localised to the ipsilateral limb to move away from the stimulus source (Hagbarth, 1960). Likewise, Grimby (1963b) used electromyography recordings in healthy adult volunteers to fully describe the general pattern of reflex EMG activity in the short hallux flexor and extensor following noxious electrical stimulation to the plantar surface of the foot. These studies confirmed that the muscle activated in humans were similar to those described by Sherrington in vertebrate animals, and the latencies short enough to indicate that the components of the reflex formed a spinal reflex arc.

Many researchers use the flexion withdrawal reflex as an objective indicator of experimental pain in humans. The flexion withdrawal reflex is specifically evoked by high-threshold, noxious stimulation, and the reflex threshold is equivalent to that required for pain perception in adult man (Willer, 1977). Indeed, reflex response EMG parameters such as latency to response and magnitude of activity have been shown to be dependent on stimulus intensity. Studies examining effect of various stimulus intensities on reflex activity show increasing stimulus strength accompanies decreases in latency to reflex activity, larger reflex amplitude

and longer duration of response (Grimby, 1963a; Shahani *et al.*, 1971). Increasing stimulus intensity is also correlated with subjective pain rating (Neziri *et al.*, 2009a; Willer, 1977).

3.1.2 Flexion withdrawal reflex in the neonate

3.1.2.1 Cutaneous sensory thresholds are lower in the neonate

In the animal and human developing nervous system, the neural circuitry underlying the flexion withdrawal reflex is immature and subject to substantial refinement in early life. Unlike the adult, neonates exhibit low sensory thresholds, larger receptive fields with exaggerated and poorly coordinated motor movement. Previous studies show that flexion withdrawal reflex activity can be evoked by low-level innocuous intensity stimuli to the foot and cutaneous thresholds rise with increasing age (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). As with the adult, cutaneous sensitivity increases even further by local skin damage (Abdulkader *et al.*, 2008a; Fitzgerald *et al.*, 1988a; Fitzgerald *et al.*, 1989). The characteristics of heightened sensitivity to innocuous stimulation in the neonate indicate that the flexion withdrawal is not a nociceptive-specific response, in contrast to the reflex properties in the adult.

3.1.2.2 Visual observations of flexion withdrawal reflex activity

Much of the work investigating flexion withdrawal reflex activity in the human neonate has depended upon visual observations of behaviour. Anecdotal evidence of body movement following a heel lance first described the infant response as 'immediate withdrawal of both legs followed by crying… [and] vigorous gross motor activity' (Franck, 1986). Early work by Fitzgerald and coworkers (1988b) studying cutaneous sensitivity used behavioural observations to demonstrate that the threshold for flexion withdrawal was lowest in the youngest of infants, and gradually increased with gestational age; this work was conducted in parallel with rat pups across various ages and complemented the animal data. Here, these authors defined flexion withdrawal in both species as 'clear, withdrawal of the leg [hind limb], not simply a twitch of the toes or flexion of the foot'. Andrews *et al* (1994) went on to use the same definition of visual withdrawal to demonstrate that reflex receptive fields in the infant are large at younger ages and become more refined with postnatal development. Older infants exhibited maximal sensitivity when stimuli were applied on the plantar surface of the foot and minimal sensitivity in the buttock; younger infants by contrast exhibit uniform sensitivity across the whole limb. Further work by the same laboratory used abdominal flexor reflex

studies to show that leg reflex movement was uncoordinated in the youngest infants, incorporating activity from both limbs, and gradually decreased with age until the reflex was unilateral and then absent (Andrews *et al.*, 2002a; Andrews *et al.*, 2002b). Collectively these observations provide useful information of cutaneous sensitivity, reflex receptive fields and motor coordination by detecting when leg movement occurs and does not occur following cutaneous stimulation, but these measures are subjective in nature and lack comprehensive detail of efferent motor activity such as latency or size of response.

3.1.2.3 Direct recordings of motor reflex activity in human infants

Direct recordings of motor activity have been used to investigate the reflex properties in the human infant, although these studies are few and far between. Rowlandson *et al* (1985) examined cutaneous reflex EMG responses from the lower limb (tibialis anterior) following electrical stimulation of the toes in children aged 1 – 16 years of age. The findings demonstrated the pattern of reflex EMG activity developed over time, most significantly over the first 2 years of age as all the elements of an adult reflex response were recognised at this point, and fully matured by 8-12 years of age. This is due to significant changes in the organisation of spinal circuitry underlying the reflex over this period of development.

More recent work, by Andrews *et al* (1999), examined the effect of increasing forces of mechanical stimuli to the heel on biceps femoris activity using surface EMG recordings in the infant; the aim was to establish the stimulus-response characteristics of motor reflex activity. In agreement with adult reflex excitability, increasing stimulus intensities led to an increase in the magnitude of flexor (biceps femoris) reflex activity accompanied with a decrease in the latency to onset of the response in the infant. Andrews *et al* (1999) established that reflex activity could be measured in the infant using surface EMG recordings, even after a noxious heel lance, and furthermore, a noxious heel lance produced the largest flexion withdrawal reflex response. This was the first study to provide quantitative analysis of flexion withdrawal reflex activity at various mechanical intensities (including noxious stimulation) in the human infant. Thus indicating the usefulness of the flexion withdrawal reflex and the relative quantitative measurement in noxious and non-noxious evoked responses in the neonate. Corresponding observations of behaviour have shown that increasing stimulus intensities lead to activation of other components of the motor system. Abdulkander and colleagues (2008a) examined the sequence of body responses to graded stimulation using von Frey hair application to the heel in preterm and full-term infants. Flexion withdrawal reflex activity was the first motor response to be evoked, followed by alternating responses such as eye opening

and gross body movements at larger forces, until extreme facial responses such as grimacing and cry were observed with largest intensities of stimulation (Abdulkader *et al.*, 2008a).

Other studies have examined development of reflex properties in the neonate using direct measurement of muscle activity, although specific activity of the lower limb has not been the response of interest. Issler *et al* (1983) examined cutaneous reflex responses from forearm flexor and extensor muscles following electrical stimulation of the finger in infants aged 6 weeks post-natal age to 11 years. EMG recordings indicated that newborn infants exhibit an exaggerated short-latency component that disappears with after 3 years age to be replaced by a more adult-like pattern. The authors postulate the delayed functional development of corticospinal tracts, which modulate spinal cord activity, may contribute to the enhanced excitability at younger ages. In 1991, the development muscle coordination of the stretch reflex, which is a monosynaptic spinal reflex, was investigated in infants aged 31 weeks GA to 55 years using surface EMG recordings of upper limb motor activity (O'Sullivan *et al.*, 1991). Whilst confirming that the threshold for evoking a reflex response increased with gestational age, the authors found that evoked responses were most radial along the muscles of the upper limb at birth and included antagonistic muscles, and progressively became more refined over 2-4 years of age. Again, this may be due to maturation of the underlying reflex circuitry and additionally the progress of synaptic elimination at the level of the motorneurons. It is clear the reflex activity, albeit polysynaptic or monosynaptic, is subject to a developmental progression that must be apparent in the human following a noxious stimulus.

3.1.2.4 Coordination of muscle activity is poorly directed in neonates

Indeed, our understanding of flexion withdrawal in the animal is far more comprehensive than that of the human infant due to the feasibility of obtaining direct recordings of muscle activity together with other components of spinal reflex circuitry and also with observations of nocifensive activity.

Animal and human studies shown that in contrast to adult behaviour, observations of neonatal lower limb activity at low-level intensity consist uncoordinated gross movement of all limbs in response to stimulation in the kitten (Ekholm, 1967), rat-pup (Holmberg *et al.*, 1996) and human infant (Andrews *et al.*, 2002b; Franck, 1986). Reflexes are exaggerated and this is reflected by electrophysiological recordings of dorsal horn activity which continue to fire after single stimulation for a longer period of time in younger rat pups than in the adult (Fitzgerald

et al., 1984). The direction of reflex movement is also unfocused early in life. In neonatal rats, thermal stimulation to one-side of the rat tail (using focused lasers) evokes a high error rate of tail-flick responses with movement directed towards the stimulus that is more frequent between P0 to P10, and gradually improves over the first 3 postnatal weeks (Waldenstrom *et al.*, 2003).

3.1.3 Clinical pain assessment

In the neonatal intensive care unit, clinicians need to be able to gauge how much pain an infant is feeling and how much analgesia is required. Current clinical pain assessment tools necessarily rely on behavioural and physiological measures in the neonate. This is due to the inability of infants to accurately communicate their pain experience. Facial actions are an important emotional response to pain. Facial expression is the most specific and consistent pain response (Grunau *et al.*, 1998; Grunau *et al.*, 1987). Cortical haemodynamic activity correlates well with the facial expression components of the PIPP scoring system (Slater *et al.*, 2008) and suggests that nociceptive pathways from the spinal cord conveying information to the somatosensory cortex reliably activate brainstem nuclei responsible for facial expression. However, we do not know how well they correlate with nociceptive flexion withdrawal reflex activity. Investigations of behavioural expressions of pain are warranted to establish their usefulness in this population, and so the development of the underlying central nervous system sensory circuits and motor pathways must be considered.

3.1.4 Surface EMG measurement of flexion withdrawal reflex activity

EMG is used understand the biomechanics of motor movement and has been used extensively for the measurement of lower limb activity during flexion withdrawal. Hagbarth (1960) was the first to provide objective analysis of spinal flexion withdrawal reflex activity in the human and facilitated this with EMG recordings of motor activity. Electrical activity generated by a muscle can be recorded with electrodes placed over the skin. The resulting signal is the sum of the action potentials generated by pools of motor neurons innervating the muscle. Greater amplitude of EMG activity is indicative of greater motor unit activation. Several features of the EMG recording including onset latency, amplitude and area under the curve have been used as an index of neural drive from the spinal cord and provide quantification of the motor response (Farina *et al.*, 2010; Sandrini *et al.*, 2005)

3.2 Aim of the chapter

The aim of this chapter was to study the postnatal development of the nociceptive flexion withdrawal reflex in the human infant using surface electromyographical (EMG) recordings from the lower limb. The key objectives were:

1) To examine the characteristics of surface EMG recordings as a measure of nociceptive-specific flexion reflex withdrawal activity in the preterm and full-term infant
2) To investigate whether the nociceptive flexion withdrawal reflex changes with gestational age
3) To investigate the specificity of flexion withdrawal reflex activity to a noxious stimulus
4) To test the laterality of the nociceptive flexion withdrawal reflex and whether that changes with age
5) To test whether there is a relationship between nociceptive flexion withdrawal reflex activity and observed changes in facial motor activity in the preterm and full-term infant

3.3 Methods

3.3.1 Participants

All the participants were in-patients on the Neonatal Intensive Care Unit (NICU), Special Care Baby Unit (SCBU), Transitional Care (TC) and Post-natal ward at UCLH. The participant criteria were as described in the General Methods (see section 2.2).

Sixty-five studies were conducted in infants aged 28 to 43 weeks gestational age (GA). Gestational age (or postmenstrual age) is the time elapsed between the first day of the last normal menstrual period and the day of delivery. Four infants were studied on multiple test occasions: Infant 11 was studied three times (32.71, 34.71 & 39.29 weeks GA), Infant 20 was studied twice (35.00 & 36.00 weeks GA), Infant 25 was studied twice (35.57 & 38.86 weeks GA) and Infant 30 was studied twice (36.43 & 37.00 weeks GA). The individual infant characteristics are described in Table 3.2-1.

Infant No.	Sex	Multiple births	GA at birth (weeks)	GA at study (weeks)	PNA (days)	Weight at birth (g)	Weight at study (g)	Reason for admission
1	Female	Triplet	27.00	28.43	10	1079	1062	Extreme prematurity
2	Male	Triplet	27.00	28.57	11	1060	1018	Extreme prematurity
3	Male	Singleton	25.71	30.71	35	796	1094	Extreme prematurity
4	Female	Twin	29.14	31.43	16	1182	1300	Prematurity
5	Female	Singleton	30.29	31.86	11	1274	1274	Prematurity
6	Male	Singleton	30.71	31.86	8	1519	1477	Prematurity
7	Female	Twin	30.86	32.29	10	1610	1460	Prematurity
8	Female	Twin	30.86	32.29	10	1585	1500	Prematurity
9	Male	Singleton	23.43	32.57	64	447	850	Extreme prematurity
10	Female	Singleton	31.71	32.57	6	1053	930	Prematurity
11*	Male	Singleton	24.86	32.71; 34.71; 39.29	55; 69; 101	780	1622; 1658; 2658	Extreme prematurity
12	Male	Singleton	32.00	32.71	5	1800	1670	Prematurity
13	Male	Twin	32.00	34.00	14	1732	1818	Prematurity
14	Female	Twin	32.00	34.00	14	1544	1656	Prematurity
15	Male	Singleton	31.57	34.00	17	1450	1716	Respiratory Distress Syndrome
16	Female	Singleton	27.29	34.43	50	1080	1375	Extreme prematurity
17	Male	Twin	33.43	34.57	8	1836	1835	Prematurity
18	Male	Singleton	34.14	34.57	3	2160	2008	Prematurity
19	Female	Twin	34.43	35.00	4	2120	1989	Prematurity

Infant No.	Sex	Multiple births	GA at birth (weeks)	GA at study (weeks)	PNA (days)	Weight at birth (g)	Weight at study (g)	Reason for admission
20*	Male	Triplet	32.14	35.00; 36.00	20; 27	1382	1582; 1886	Prematurity
21	Male	Singleton	35.14	35.14	0	2580	2580	Monitoring blood sugar level
22	Male	Twin	33.57	35.14	11	1940	2115	Prematurity
23	Male	Twin	34.86	35.29	3	1840	1747	Prematurity
24	Female	Singleton	33.43	35.43	14	2030	1978	Prematurity
25*	Female	Singleton	25.57	35.57; 38.86	70; 93	767	1538; 2095	Extreme prematurity
26	Female	Twin	35.29	35.86	4	2076	2055	Monitoring blood sugar level
27	Female	Twin	35.29	35.86	4	1694	1701	Prematurity
28	Male	Twin	36.00	36.14	1	2350	2350	Temperature instability
29	Male	Singleton	35.71	36.29	4	2700	2780	Monitoring blood sugar level/ jaundice
30*	Female	Singleton	35.86	36.43; 37.00	4; 8	2678	2480; 2374	Neonatal Abstinence Syndrome
31	Male	Singleton	35.14	36.43	9	1540	1529	Prematurity
32	Male	Singleton	36.14	37.00	6	2014	1969	Tachyapnoea
33	Female	Triplet	31.86	37.57	40	1396	2440	Prematurity
34	Female	Twin	24.00	38.00	98	540	1696	Extreme prematurity
35	Female	Singleton	37.86	38.00	1	2389	2389	Jaundice
36	Male	Singleton	38.00	38.14	1	3500	3500	Jaundice
37	Female	Singleton	37.71	38.29	4	2094	1959	Monitoring blood sugar level
38	Male	Singleton	38.00	38.57	4	3040	3040	Jaundice
39	Female	Singleton	37.86	38.71	6	3084	2595	Monitoring blood sugar level/jaundice
40	Female	Singleton	38.29	38.71	3	3295	3295	Monitoring blood sugar level/ jaundice
41	Male	Singleton	38.43	38.71	2	3580	3580	Infection
42	Female	Singleton	38.00	39.00	7	3182	2800	On postnatal ward
43	Female	Singleton	38.00	39.00	7	3250	2870	Establishing feeds
44	Female	Singleton	32.57	39.43	48	1692	2744	Prematurity
45	Male	Singleton	39.00	39.71	5	3278	3060	Neonatal Abstinence Syndrome
46	Male	Singleton	24.71	40.43	110	753	2250	Extreme prematurity
47	Male	Singleton	40.00	40.43	3	3640	3640	Infection
48	Female	Singleton	41.14	40.57	4	2590	2560	Jaundice
49	Male	Singleton	40.43	40.57	1	3560	3560	Jaundice
50	Male	Singleton	40.00	40.57	4	2940	2960	Infection
51	Male	Singleton	40.57	41.00	3	3580	3380	Monitoring blood sugar level
52	Female	Singleton	40.71	41.00	2	2700	2700	Jaundice
53	Male	Singleton	40.00	41.00	7	3570	3390	Jaundice
54	Male	Twin	24.86	41.43	116	600	1923	Extreme prematurity

Infant No.	Sex	Multiple births	GA at birth (weeks)	GA at study (weeks)	PNA (days)	Weight at birth (g)	Weight at study (g)	Reason for admission
55	Male	Singleton	40.43	41.43	7	3370	3038	Establishing feeds
56	Female	Singleton	41.43	41.57	1	3950	3950	On postnatal ward
57	Male	Singleton	40.86	41.57	5	4420	4002	Meconium observations
58	Male	Singleton	40.57	41.71	8	2500	2605	Respiratory Distress Syndrome
59	Female	Singleton	41.43	41.71	2	3186	3180	Infection
60	Male	Singleton	41.57	42.29	5	3380	3290	Establishing feeds

Table 3-1: Individual characteristics of infants included in the study
GA, gestational age; PNA, postnatal age. * Infants who were studied on more than one occasion: Infant no. 11 was studied three times; infants 20, 25 and 30 were studied twice.

3.3.2 Study design

A clinically required noxious heel lance and non-noxious touch stimulus were performed on the heel at the same site in each infant as described in the General Methods (section 2.4). Spinal flexion reflex withdrawal activity and facial behaviour evoked by these stimuli were measured using time-locked surface EMG and video recording techniques respectively. Figure 3-1 illustrates the experimental time-line.

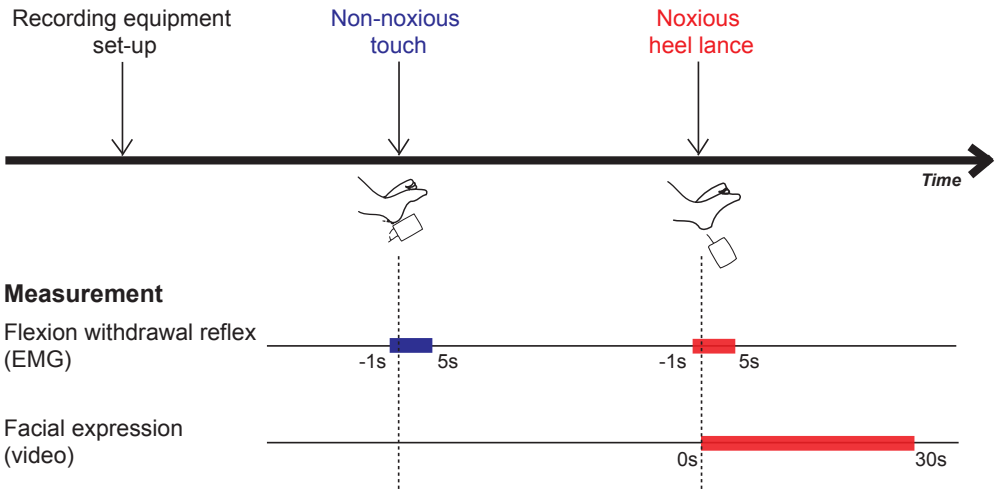


Figure 3-1: Experimental time-line
Dotted lines indicate the time at which the stimulus was performed; coloured blocks represent the recording epoch used in data analysis (blue = non-noxious touch; red = noxious heel lance).

3.3.3 Recording

The protocol for EMG and video recording are described in the General Methods (see section 2.6). Sixty-five studies were conducted with ipsilateral EMG recordings of biceps femoris muscle flexion withdrawal reflex activity. Of these, 57 studies had contralateral EMG recordings and 64 had video recordings of facial expression.

3.3.4 Data analysis

3.3.4.1 EMG analysis

EMG analysis is described in detail in the General Methods (see section 2.7.1). A clear reflex response to heel stimulation was defined as a change in EMG activity that exceeded 3SD of baseline activity. Baseline activity (-1000ms to time 0) was stable and there were no significant differences in mean baseline activity between groups. The reflex response was quantified by:

(1) Latency: *Onset latency (ms) & peak latency (ms)*

(2) The pattern of activity: *(i) Non-latency corrected & (ii) Latency-corrected analysis*

(3) Mean amplitude over 2000ms: *(i) Non-latency corrected & (ii) Latency-corrected analysis*

3.3.4.2 Facial behaviour analysis

Facial behaviour analysis is described in the General Methods (see section 2.7.2). A facial response was characterised by the presence brow bulge, eye squeeze and nasolabial furrow behavioural features. Facial behaviour was quantified using the measures below:

(1) Incidence: *number of infants expression a visable facial response (n/N; %)*

(2) Latency: *first visible facial response (s)*

(3) TFS: the duration of brow bulge, eye-squeeze and nasolabial furrow features were individually scored and combined to give a total facial score

3.3.5 Statistical analysis

Statistical analysis is described in the General Methods (see section 2.8). Significance testing was performed as follows:

(1) Latency to onset of reflex response, and peak activity, were compared also using a *Student's t-test* for (1) gestational age -preterm versus full-term (unpaired t-test), (2) stimulus specificity - noxious heel lance versus non-noxious touch stimulation (paired t-test), and (3) laterality - ipsilateral versus contralateral activity (unpaired t-test). (4) *Pearson correlation analysis* was performed to test the relationship between gestation age and peak latency.

(2) The pattern of activity was compared for (1) gestational age - preterm versus full-term infants, (2) stimulus specificity- a noxious heel lance versus non-noxious touch stimulation of the heel, (3) laterality- ipsilateral versus contralateral biceps femoris activity using a *two-way analysis of variance (ANOVA) with repeated measures and Bonferroni post-hoc* testing over the recording time period. (4) The association between gestational age, stimulus type and incidence of flexion withdrawal reflex activity was tested using *Fisher's exact test*.

(3) The mean activity over 2000ms was compared using a *Student's t-test* for (1) gestational age - preterm versus full-term infants (unpaired t-test), (2) stimulus specificity - noxious heel lance versus non-noxious touch stimulation (paired t-test), and (3) laterality - ipsilateral versus contralateral activity (unpaired t-test).

(4) Facial behaviour was compared for (1) gestational age- preterm versus full-term using a *Student's unpaired t-tes*t. (2) The association between gestational age, incidence of flexion withdrawal reflex activity and a visible facial response was tested using *Fisher's exact test*. (3) The relationship between TFS versus mean EMG activity over 2000ms, and (4) for latency to first visible facial response and peak EMG activity using *Pearson correlation analysis*.

3.4 Results

Sixty-five studies were conducted in preterm and full-term infants to assess flexion withdrawal reflex activity evoked from a single noxious lance and non-noxious touch to the heel.

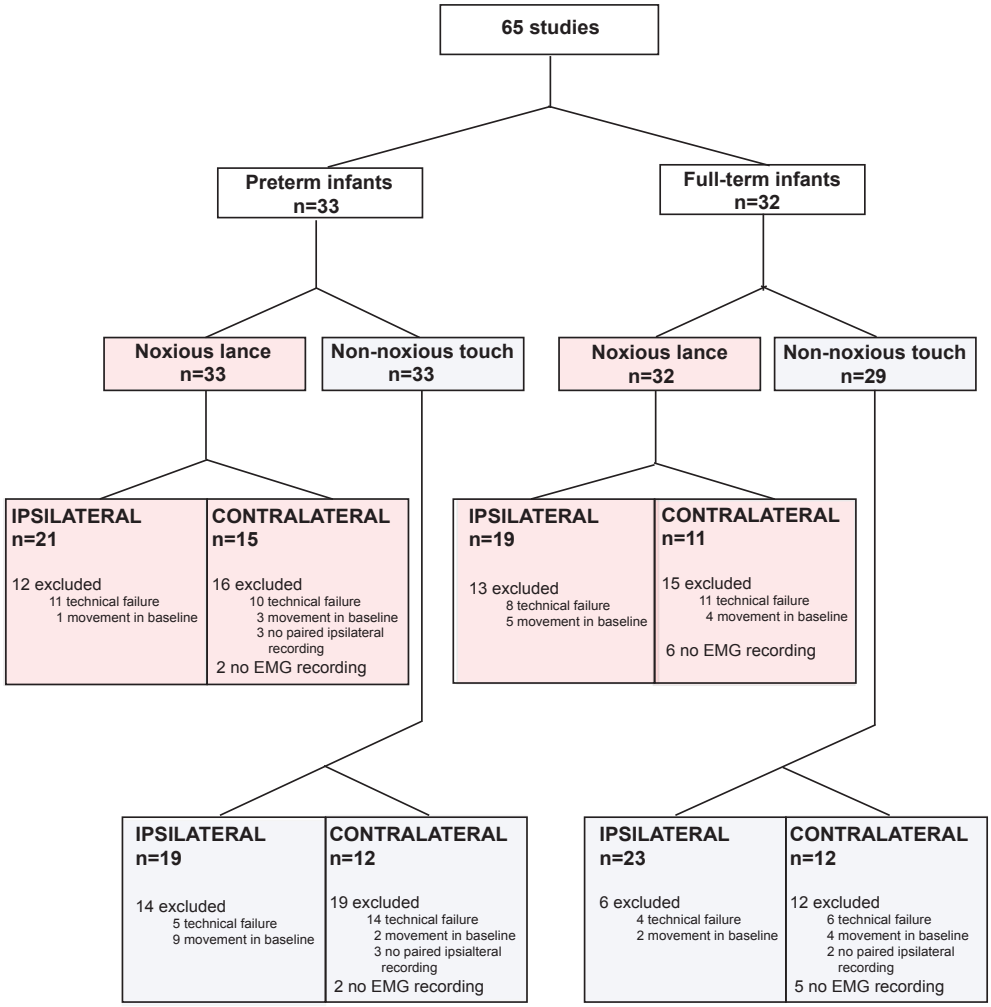


Figure 3-2: Identification of recordings included in EMG analysis
EMG recordings were excluded from the analysis for technical failure and movement in the baseline using the criteria described in methods (see section 2.7.1 on page 70).

3.4.1 Nociceptive withdrawal reflex EMG characteristics in preterm and full-term infants

This section focuses on the characterisation of flexion withdrawal reflex EMG activity in preterm and full-term infants following a noxious heel lance. Forty studies were included in the final EMG analysis: 21 preterm and 19 full-term infants (Figure 3-2). Infant demographics of each age group are in Table 3-2.

	Preterm Infants (N=21)	**Full-term Infants (N=19)**
Male; (n/N)	57%; (12/21)	53% (10/19)
Mean GA at birth (weeks)	31.23±3.94; range 23.43-36.00	35.60±5.23; range 24.71-41.57
Mean GA at study (weeks)	34.21±1.94; range 30.71-36.43	39.07±1.33; range 37.00-42.29
Mean PNA (days)	20.86±21.18; range 1-69	24.26±36.56; range 1-110
Mean weight at study (g)	1726.57±488.33 range 850.0-2780.0	2782.58±508.56; range 1959.0-3580.0
Right heel stimulated; (n/N)	38%; (8/21)	47% (9/19)

Table 3-2: Infant demographics
Data given as mean±SD unless otherwise stated.

Initially, EMG recordings were characterised in full-term infants because these were a more robust age group. EMG data analysis techniques were optimised on this age group (see section 2.7.1) prior to implementing the same analysis on the less stable, preterm infant group. Example EMG recordings following a noxious heel lance are shown in Figure 3-3 on page 91. Finally comparisons in the flexion withdrawal reflex motor activity were made between preterm and full-term age groups.

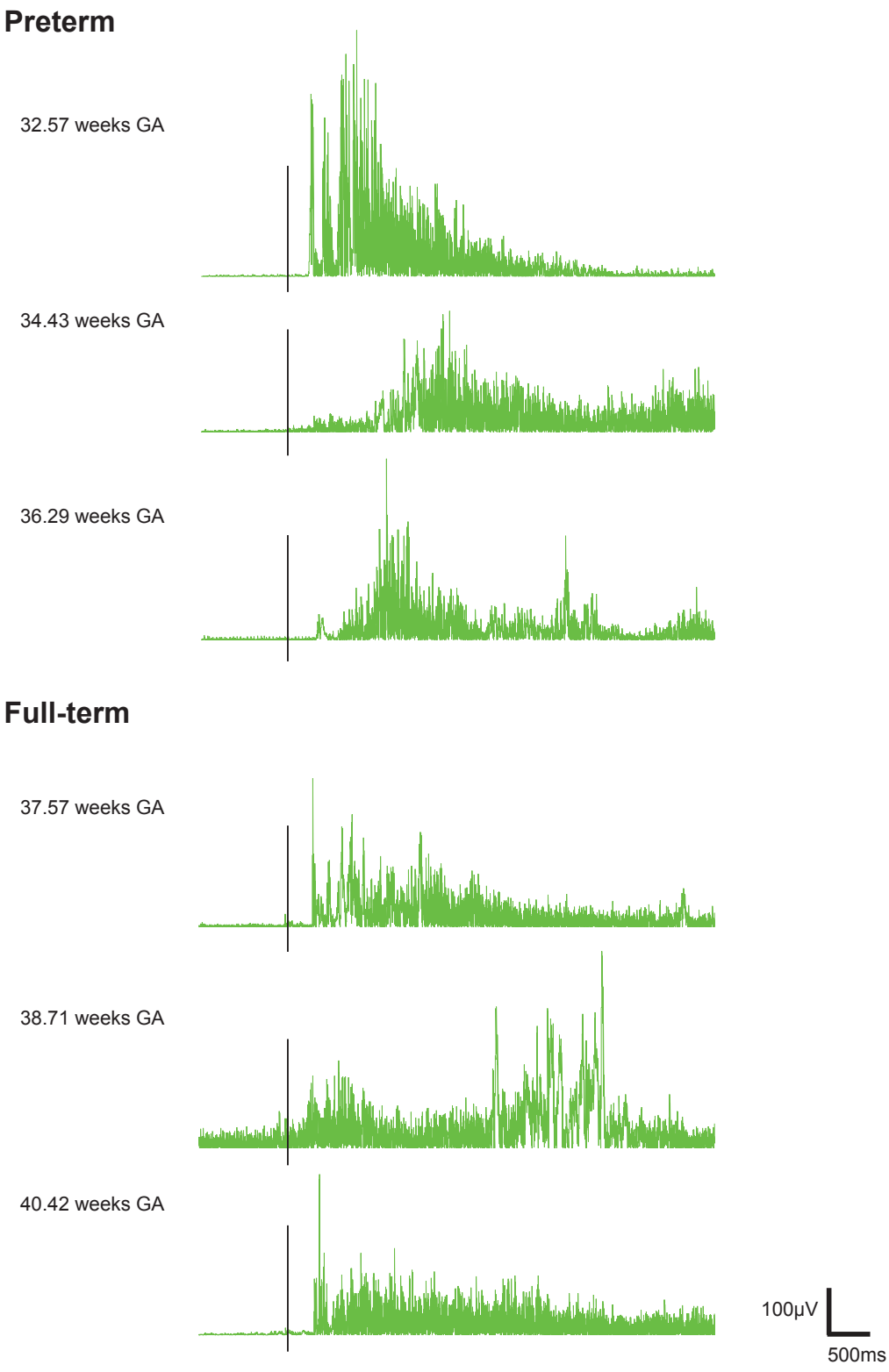


Figure 3-3: Example EMG recordings from preterm and full-term infants following a noxious heel lance
Vertical black line indicates the time of heel lance. The scale bar is given in the bottom right corner.

3.4.2 Characterisation of flexion withdrawal reflex activity in full-term infants

3.4.2.1 Latency

All full-term infants exhibited a clear flexion withdrawal reflex response to a noxious heel lance (n=19). The mean latency to onset of flexion withdrawal reflex activity was 369.1ms (95% CI 277.3-460.9ms). The mean time taken to peak amplitude was 934.2ms (95% CI 742.2-1126.0ms). The variation in absolute latency values for the two measures is shown in Figure 3-4.

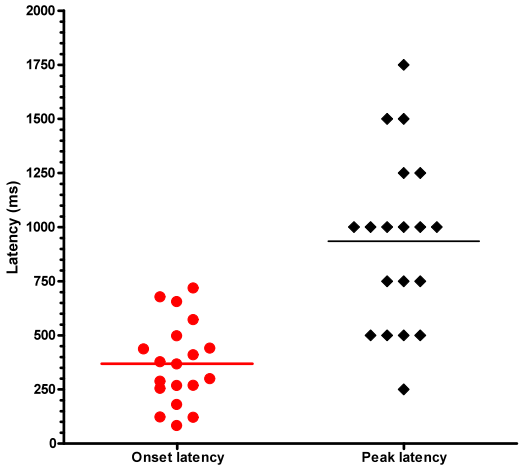


Figure 3-4: Variability in onset latency and peak latency in full-term infants
Each point represents an individual infant; horizontal line indicates the group mean.

3.4.2.2 Pattern of reflex activity: (1) Non-latency corrected

The pattern of reflex activity was quantified in real time using non-latency corrected analysis. The RMS of 250ms time-bins were calculated for each infant to identify how the pattern of flexion withdrawal reflex activity changed over time. The pattern of activity for each individual full-term infant is shown in Figure 3-5.

The mean pattern of activity for the entire group of full-term infants is shown in Figure 3-10 on page 97. Flexion withdrawal reflex EMG activity rapidly increased in the first 1000ms after the stimulus. Mean peak activity was 43.94μV (95% CI 30.20-57.67μV) and occurred 750-1000ms after the stimulus. Subsequent activity decreased over the following 1000ms by 41% of peak activity to 25.89μV (95% CI 20.33-31.41μV) at 2000ms, before a second wave of motor activity occurred. A clear offset of the reflex could not be identified; evoked activity

was sustained above 20μV for the duration of the 5s post-stimulus period and did not return to baseline levels.

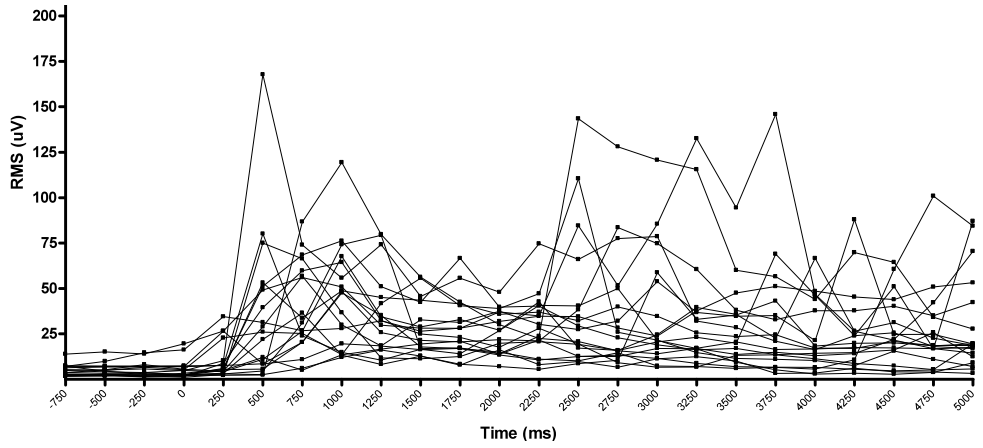


Figure 3-5: Individual full-term infant ipsiateral biceps femoris activity following a noxious heel lance
Ipsilateral biceps femoris activity was calculated using the root mean square (RMS) of 250ms time bins i.e. -750 = activity between -1000 and -750ms. The noxious stimulus was applied at time 0.

3.4.2.3 Pattern of activity: (2) Latency-corrected

The same EMG recordings were also analysed using a method that corrects for variability in onset latency. In this way, the recordings were aligned from the time of onset of activity and then analysed in 250ms time bins as above. The time of onset was defined as the time when activity exceeded 3 SD of mean baseline. This method permits the characteristics of the actual reflex activity to be determined. Latency-corrected analysis of the group mean is shown in Figure 3-11 on page 97. Corrected peak activity was 47.30μV (95% CI 29.32-65.27μV) and occurred in the first 250ms of the reflex response. Flexion withdrawal reflex activity subsequently decreased by 45% over the following 1500ms before a second wave of motor activity occurred. Evoked activity was sustained above 20μV for the duration of the 5s post-stimulus period and did not return to baseline levels.

Overall correcting for latency had little effect on the gross pattern of EMG activity compared to non-latency corrected analysis. However, the former method had the advantage of accounting for the variability in onset latency and better represented evoked reflex activity. From now on, the time course of EMG activity will be *solely* analysed using latency-corrected methods.

3.4.3 Characterisation of the nociceptive withdrawal reflex in preterm infants

3.4.3.1 Latency

All preterm infants exhibited a clear flexion withdrawal reflex response (n=21). The mean latency to onset of flexion withdrawal reflex activity was 416.5ms (95% CI 310.3-522.8ms).The mean time taken to peak amplitude was 1214.0ms (95% CI 1037.0-1392.0ms). The variability of latency values for the two measures is shown in Figure 3-6.

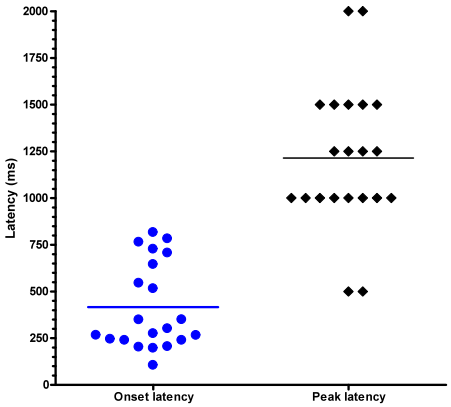


Figure 3-6: Variability in onset latency and peak latency in preterm infants
Each point represents an individual infant; horizontal line indicates the group mean.

3.4.3.2 Pattern of activity: Latency-corrected

The pattern of activity for each individual preterm infant (Figure 3-7) and the group average (Figure 3-10 on page 97) when non-latency corrected are shown for illustrative purposes.

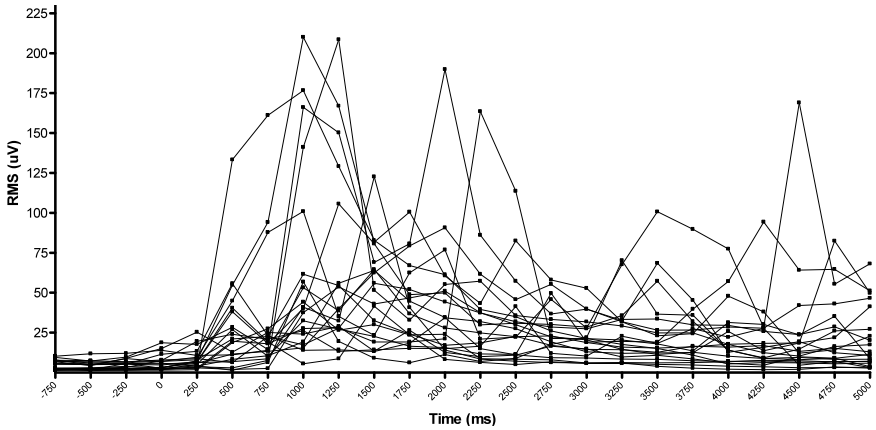


Figure 3-7: Preterm individual ipsiateral biceps femoris activity following a noxious heel lance
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins. The noxious stimulus was applied at time 0.

Latency-corrected analysis was used to characterise the pattern of EMG activity in preterm infants, as clarified in the previous section. The group mean is shown in Figure 3-11 on page 97. Corrected peak activity was 64.24μV (95% CI 36.39-92.09μV) at 750-1000ms from the onset of the reflex response. Flexion withdrawal reflex activity steadily decreased until 2500-2750ms post-onset where activity was maintained at a constant, raised level until the end of the recording window. As with full-term infants, termination of the reflex activity could not be clearly identified; evoked activity was sustained above 17μV for the duration of the 5s post-stimulus period and did not return to baseline levels.

3.4.4 Comparison of flexion withdrawal reflex activity in preterm and full-term infants

The properties of the flexion withdrawal reflex evoked by a noxious heel lance were compared between the two groups of infants: full-term (n=19) and preterm infants (n=21).

3.4.4.1 Latency to peak activity is longer in younger, preterm infants

There were no significant differences in onset latency between preterm infants [416.5ms (95% CI 310.3-522.8ms)] and full-term infants [369.1ms (95% CI 277.3-460.9ms)]; unpaired t-test, p=0.49. The distribution of data tested positive for normality. However, time to peak activity was significantly longer in preterm infants [1214.0ms (95% CI 1037.0-1392ms)] compared to full-term infants [934.2ms (95% CI 742.2-1126.0ms)]; unpaired t-test, p=0.03. To test if the differences in peak latency were particularly prevalent at a specific time in gestation, a correlation analysis was performed between these variables for individual infants (Figure 3-8). Despite the negative relationship between peak latency and gestational age a correlation analysis was unable to prove the relationship significant, (R^2=0.07; p=0.11).

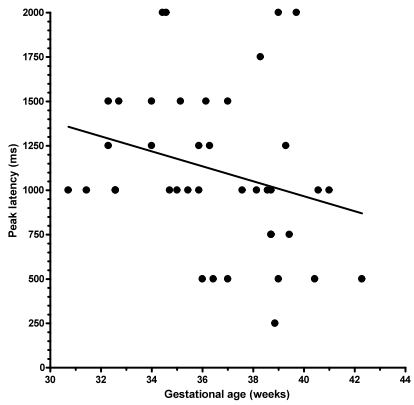


Figure 3-8: The relationship between latency to peak amplitude and gestational age
This relationship is not significant (r=-0.27, p=0.11). Each point represents an individual infant.

3.4.4.2 Differences in the pattern of reflex activity between preterm and full-term infants

Visual inspection of the pattern of activity between age groups highlighted the heightened motor activity evoked in preterm infants over the first 2000ms from onset of the reflex response compared to full-term infants (latency-corrected; Figure 3-11). There were no significant differences in the pattern of activity ($F_{1,836}$=0.01, p=0.90) due to large variation in the response between individuals (particularly in the younger preterm group-see error bars).

To test if there were significant differences in magnitude of EMG activity over the first 2000ms from the onset of the reflex response, the mean (RMS) activity over 2000ms (latency-corrected) was determined. Preterm infants evoked a mean activity of 51.71µV (95% CI 36.90-66.51µV), that was not significantly different to full-term infants, 37.99µV (95% CI 29.76-46.71µV); unpaired t-test, p=0.11.

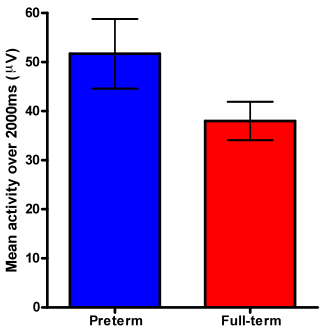


Figure 3-9: Noxious-evoked EMG activity measured over 2000ms post-onset is not significantly different between preterm and full-term infants

Non-latency corrected

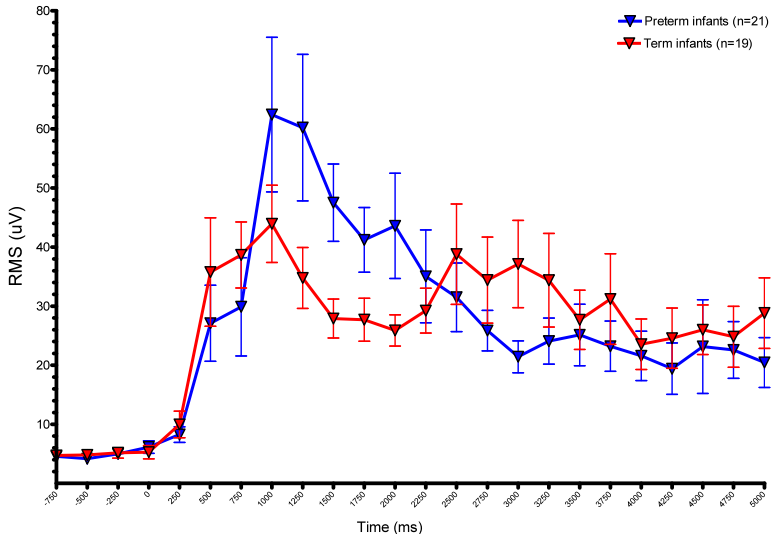


Figure 3-10: Preterm (n=21) and full-term (n=19) mean ipsilateral biceps femoris activity following a noxious stimulus (non-latency corrected analysis)
The RMS±(standard error) of each time bin is shown i.e. -750 = activity between -1000ms and -750ms.

Latency-corrected

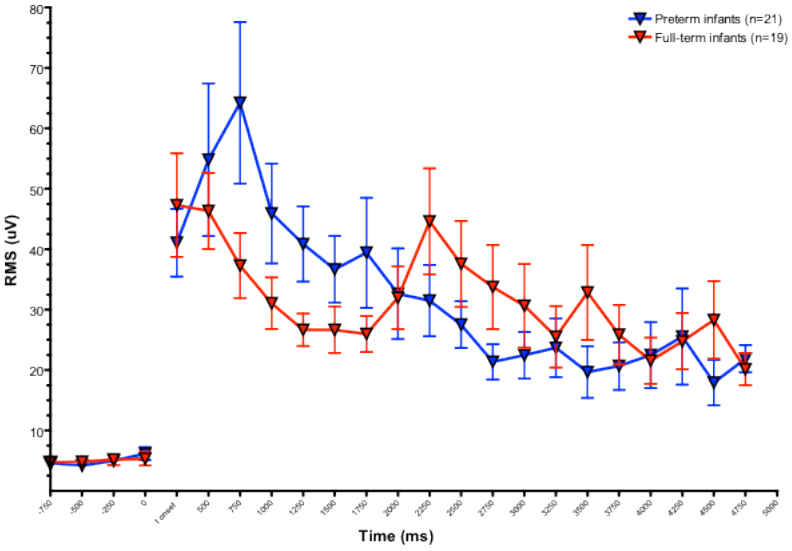


Figure 3-11: Preterm (n=21) and full-term (n=19) mean ipsilateral biceps femoris activity following a noxious stimulus (latency corrected analysis)
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recordings epoch (in completed 250ms time bins). The RMS (±standard error) in each time bin is displayed.

Summary 1

A summary of the flexion withdrawal reflex properties for preterm and term infants, and the associated p-values for age-group comparison are in Table 3-3.

	Preterm infants (n=21)	Term infants (n=19)	p-value
Mean baseline activity (μV) [1]	5.15 (3.65-6.64)	5.10 (3.29-6.89)	p>0.05
Latency to response (ms) [2]	416.5 (310.3-522.8)	369.1 (277.3-460.9)	0.49
Latency to peak activity (ms) [2]	1214.0 (1037.0-1392.0)	934.2 (742.2-1126.0)	**0.03**
Latency-corrected analysis			
Peak amplitude (μV) [1]	64.24 (36.39-92.09)	47.30 (29.32-65.27)	p>0.05
Amplitude at 2000ms (uV) [1]	32.62 (16.94-48.29)	31.97 (21.02-42.92)	p>0.05
Mean activity over 2000ms (μV) [2]	51.71 (36.90-66.51)	37.99 (29.76-46.21)	0.11

Table 3-3: Summary of ipsilateral flexion withdrawal reflex activity following a noxious heel lance in preterm and full-term infants
All data expressed as mean (95% CI). [1] Two-way ANOVA with repeated measures (Bonferroni post-hoc test); [2] Student's unpaired t-test. Significant values are coloured in green.

This section has demonstrated that EMG recordings of flexion withdrawal reflex activity provide quantitative data of reflex measurement in the human infant. The characteristics of a noxious-evoked flexion withdrawal reflex have been determined in preterm and full-term infants and used to compare the age groups.

- All infants responded to a noxious heel lance.
- Preterm infants took longer to mount a reflex response as shown by the delayed latency to peak amplitude compared to full-term infants.
- Differences in the remaining parameters (onset latency, pattern of activity and mean activity over 2000ms) were not found to be significant between the age groups.

3.4.5 Non-noxious touch evoked flexion withdrawal reflex characteristics in preterm and full-term infants

This section of the chapter investigates the characteristics of flexion withdrawal reflex EMG activity in preterm and full-term infants following a single non-noxious touch to the heel. Infant demographics are summarised in Table 3-4 on page 99. Forty-two studies were included in the final analysis: 19 preterm and 23 full-term infants. EMG activity was analysed

with same methods used to quantify the nociceptive withdrawal reflex EMG characteristics in the previous section.

As with the nociceptive flexion withdrawal reflex EMG characterisation, EMG recordings were initially analysed in full-term infants following a non-noxious touch to the heel. Preterm infant EMG recordings were subsequently analysed before comparisons were made between preterm and full-term age groups. Example EMG recordings are shown in Figure 3-12 on page 100.

	Preterm Infants (N=19)	**Term Infants (N=23)**
Male; (n/N)	63%; (12/19)	52%; (12/23)
Mean GA at birth (weeks)	31.71±3.79; range 23.43-36.00	36.19±5.76; range 24.71-41.57
Mean GA at study (weeks)	34.00±1.97; range 28.57-36.43	39.77±1.43; range 37.57-42.29
Mean PNA (days)	16.05±18.96; range 0-64	25.09±39.43; range 1-116
Mean weight at study (g)	1749.05±550.95; range 850.0-2780.0	2917.17±609.60; range 1923-4002.0
Right heel stimulated; (n/N)	26%; (5/19)	61% (14/23)

Table 3-4: Infant demographics
Data given as mean± standard deviation unless otherwise stated.

3.4.6 Characterisation of non-noxious evoked flexion withdrawal reflex EMG activity in full-term infants

Nine out of twenty-three (39%) of full-term infants exhibited a reflex response (as defined as a change in EMG activity that exceeded 3SD of baseline activity) to non-noxious stimulation with the heel-lancet device. The EMG characteristics were determined for the 9 infants who responded to the stimulus.

3.4.6.1 Latency

The mean latency to onset of flexion withdrawal reflex activity was 534.6ms (95% CI 340.5-728.7ms). The mean latency to peak amplitude was 1250.0ms (95% CI 978.2-1522.0ms). The absolute latency values for the two measures are shown in Figure 3-13 on page 101.

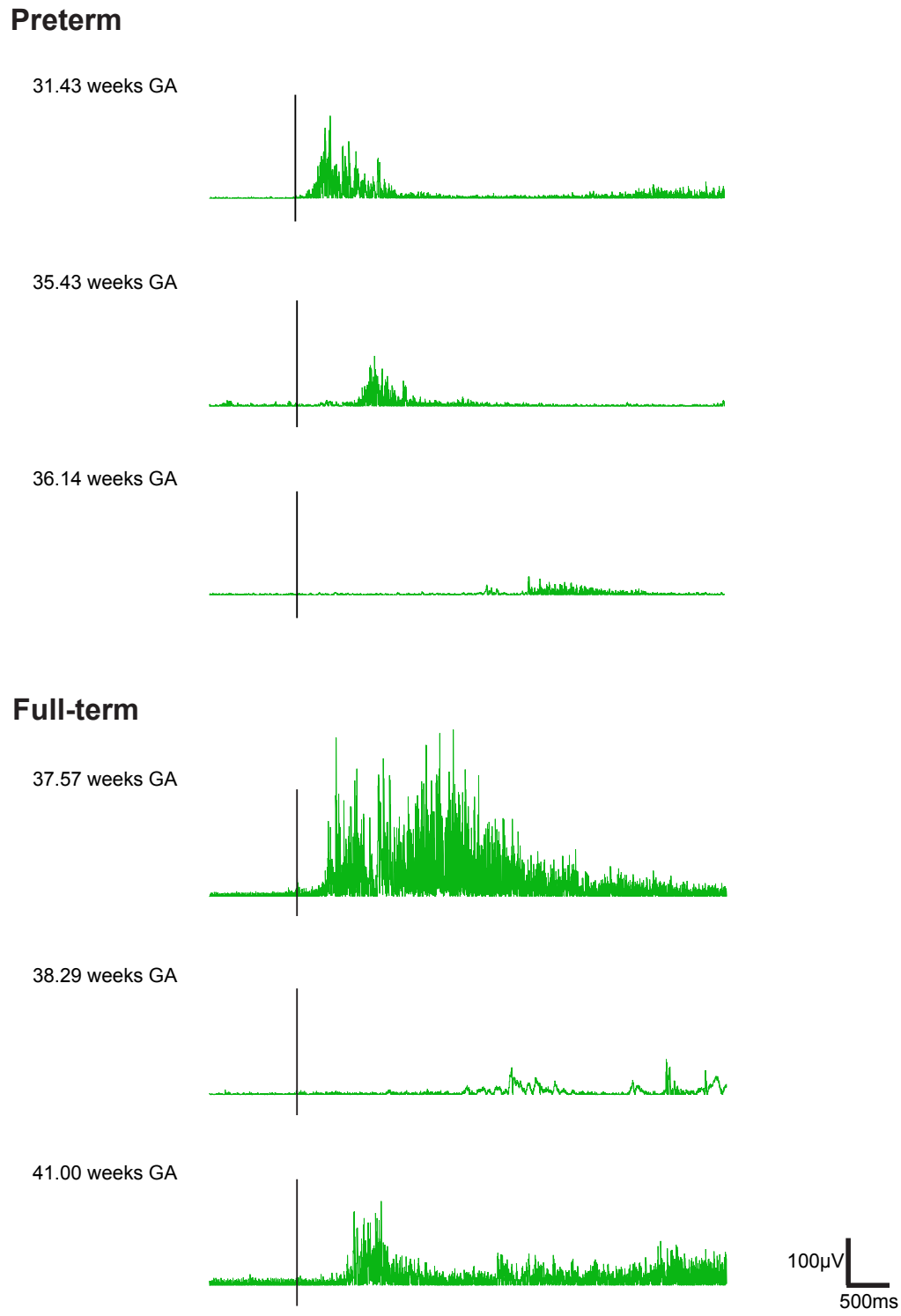


Figure 3-12: Example EMG recordings from preterm and full-term infants following non-noxious stimulation to the heel

Preterm infant at 36.14 weeks GA and full-term infant at 38.29 weeks GA did not exhibit a detectable change in EMG activity following the stimulus. All remaining infants in this figure did evoke flexion withdrawal reflex activity after the stimulus. Vertical black line indicates the time of heel lance. The scale bar is given in the bottom right corner.

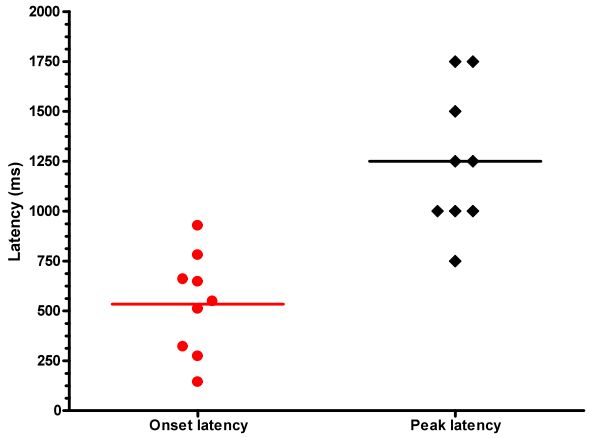


Figure 3-13: Variability in onset latency and peak latency in full-term infants who exhibit a flexion withdrawal reflex response to non-noxious touch (n=9)
Each point represents an individual infant; horizontal line indicates the group mean.

3.4.6.2 Pattern of activity: Latency-corrected

The pattern of activity for individual full-term infants who responded to the non-noxious touch stimulus (Figure 3-14) and the group average (Figure 3-17 on page 105) when non-latency corrected are shown to illustrate the pattern of activity in real time.

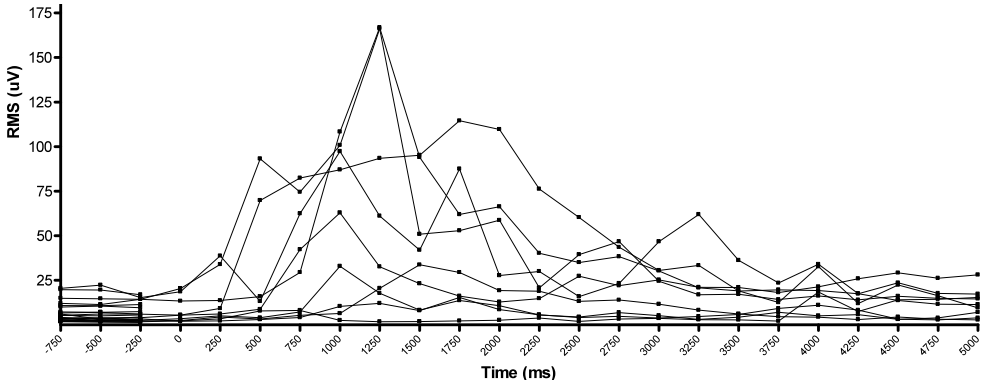


Figure 3-14: Individual ipsilateral biceps femoris activity of term infants following non-noxious touch to the heel (n=9)
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins i.e. 750ms = activity between -1000ms and 750ms. The non-noxious stimulus was applied at time 0.

EMG recordings were quantified using latency-corrected analysis to adjust for variability in onset latency. The average pattern of latency-corrected activity is shown in Figure 3-18 on page 105. Corrected peak activity occurred within the first 500ms of the reflex response and was 58.75µV (95% CI 13.28-104.20µV). Activity steadily decreased over the reminder of

the recording period, falling by 60% at 1750-2000ms into the reflex response to 23.63μV (95% CI 6.59-40.67μV).

3.4.7 Characterisation of non-noxious evoked flexion withdrawal reflex activity in preterm infants

Six out of nineteen (32%) preterm infants exhibited a flexion withdrawal reflex response to touch stimulation with the lancet device.

3.4.7.1 Latency

The mean latency to onset of flexion withdrawal reflex activity was 404.4ms (95% CI 85.06-723.8ms). The mean latency to peak amplitude was 916.7ms (95% CI 281.2-1552.0ms). The absolute latency values for the two measures are shown in Figure 3-15 on page 102.

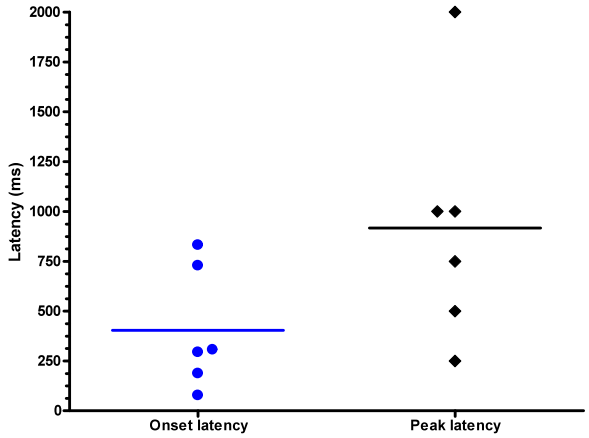


Figure 3-15: Variability in onset latency and peak latency in preterm infants who exhibit a flexion withdrawal reflex response to non-noxious touch (n=6)
Each point represents an individual infant; horizontal line indicates the group mean.

3.4.7.2 Pattern of activity

The pattern of activity for individual preterm infants (Figure 3-16) and group average (Figure 3-17 on page 105) when non-latency corrected is shown for illustrative purposes. The same EMG recordings were aligned from the time of onset of activity and analysed in 250ms time bins to correct for variability in onset latency. Corrected mean peak activity was 28.84μV (95% CI -2.04-53.87μV) at 250-500ms into the reflex response (Figure 3-18). Activity steadily decreased post-peak activity to 11.78μV (95% CI -2.21-25.78μV) at 2000ms, a decline of 59%. Evoked activity remained greater than baseline levels throughout the duration of the recording period.

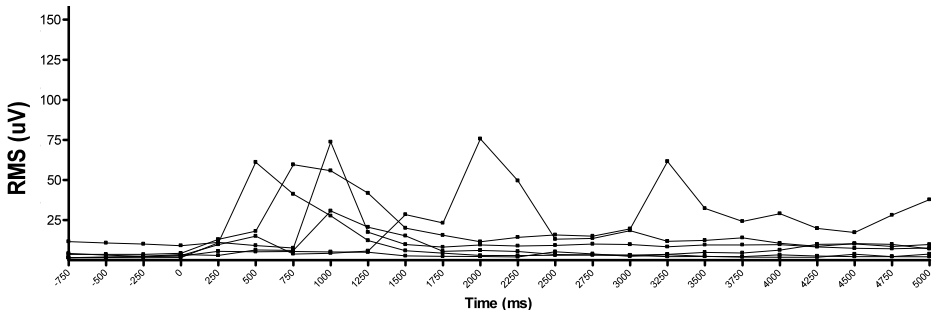


Figure 3-16 Individual ipsilateral biceps femoris activity of preterm infants with a clear reflex response following non-noxious touch
EMG activity calculated using the RMS of 250ms time bins. The stimulus was applied at time 0.

3.4.8 Comparison of non-noxious evoked flexion reflex withdrawal activity in preterm and full-term infants

The properties of the flexion withdrawal reflex evoked by a non-noxious touch stimulation of the heel were compared between full-term (n=23) and preterm (n=19) infants. The incidence of flexion withdrawal reflex activity was similar between full-term infants, 39% (n=9/23), and preterm infants, 32% (n=6/19). The association between age and incidence of non-noxious evoked reflex activity was not statistically significant (p=0.77; P1-P2= 0.04, 95% CI -0.25-0.35; Fisher's exact-test).

3.4.8.1 No differences in latency

Latency to onset of flexion withdrawal reflex activity was not significantly different between full-term and preterm infants (unpaired t-test: p=0.38). Furthermore, there was no difference in latency to peak activity between age groups (unpaired t-test: p=0.20).

3.4.8.2 No differences in the pattern of reflex activity

Infants were divided into two groups depending on whether a reflex EMG response was detected. The pattern of activity was determined using non-latency corrected analysis because the sample included EMG recordings absent of evoked activity. As Figure 3-17 on page 105 illustrates, a clear difference in the pattern of activity can be identified between infants who were responsive to the non-noxious touch stimulus and those who were not.

The pattern of reflex activity was compared between preterm and full-term infants using latency-corrected analysis (Figure 3-18; page 105). There were no differences in non-noxious evoked activity between preterm and full-term infants ($F_{1.299}$=2.47; p=0.14). In both ages, the

corrected peak activity occurred in the first 500ms of the reflex response. Corrected peak activity was smaller in the preterm group, 28.84μV (95% CI -2.04-53.87μV), compared to the full-term infants, 58.75μV (95% CI 13.28-104.20μV); this did not reach significance.

3.4.8.3 Mean activity over 2000ms

Mean activity over 2000ms was calculated from the onset of the reflex response to summarise flexion withdrawal reflex activity. There were no significant differences between preterm infants, 24.19μV (95% CI 11.28-37.11μV), and full-term infants, 45.45μV (95% CI 17.44-73.45μV); unpaired t-test: p=0.20.

Summary 2

A non-noxious touch stimulation of the heel with a lancet-device evoked flexion withdrawal reflex activity in both preterm and full-term infants. The incidence of a reflex response occurred on less than 40% of test occasions and this was independent of gestational age. A summary of the flexion withdrawal reflex properties for preterm and full-term infants, and the associated p-values for age-group comparison are in Table 3-5. Of the infants who exhibited flexion withdrawal reflex activity to a non-noxious stimulus, there were no significant differences in the EMG characteristics, including latency, pattern of activity and mean activity over 2000ms, between younger preterm and older, full-term infants. The next section investigates the sensitivity to both noxious and non-noxious stimuli in these infants further.

	Preterm infants (n=6)	Term infants (n=9)	p-value
Mean baseline activity (μV) [1]	3.88 (0.42-7.35)	7.60 (2.46-12.74)	0.23
Latency to response (ms) [2]	404.4 (85.06-723.8)	534.6 (340.5-728.7)	0.38
Latency to peak activity (ms) [2]	916.7 (281.2-1552.0)	1250.0 (978.2-1522.0)	0.20
Latency-corrected analysis			
Peak amplitude (μV) [1]	28.84 (-2.04-53.87)	58.75 (13.28-104.20)	>0.05
Amplitude at 2000ms (uV) [1]	11.78 (-2.21-25.78)	23.63 (6.59-40.67)	>0.05
Mean activity over 2000ms (μV) [2]	24.19 (11.28-37.11)	45.45 (17.44-73.45)	0.20

Table 3-5: Summary of flexion withdrawal reflex activity following non-noxious touch to the heel in preterm and full-term infants.
All data expressed as mean (95% CI). [1] Two-way ANOVA with repeated measures); [2] Student's unpaired t-test.

Non-latency corrected analysis

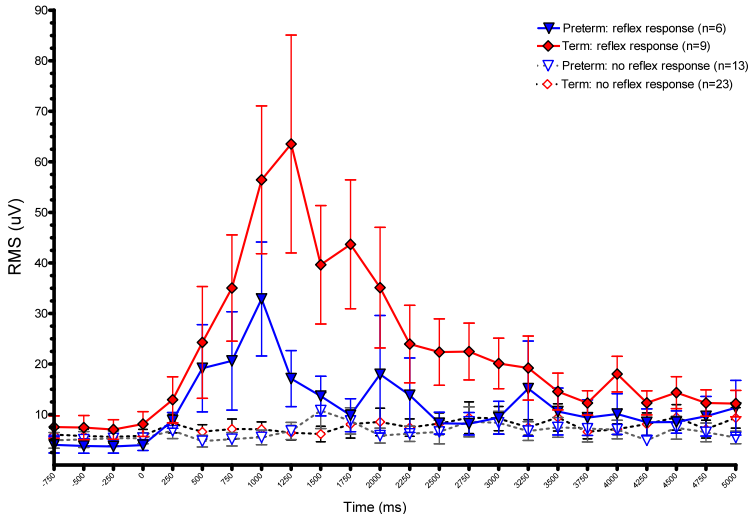


Figure 3-17: Preterm and full-term mean ipsilateral biceps femoris activity following non-noxious stimulation

Infants were divided into two groups depending on whether a reflex EMG response was detected following non-noxious stimulation of the heel. Infants with a reflex response are represented with the coloured shapes (preterm, n=6; full-term, n=9). The RMS±(standard error) of each time bin is shown i.e. -750 = activity between -1000ms and -750ms.

Latency-corrected analysis

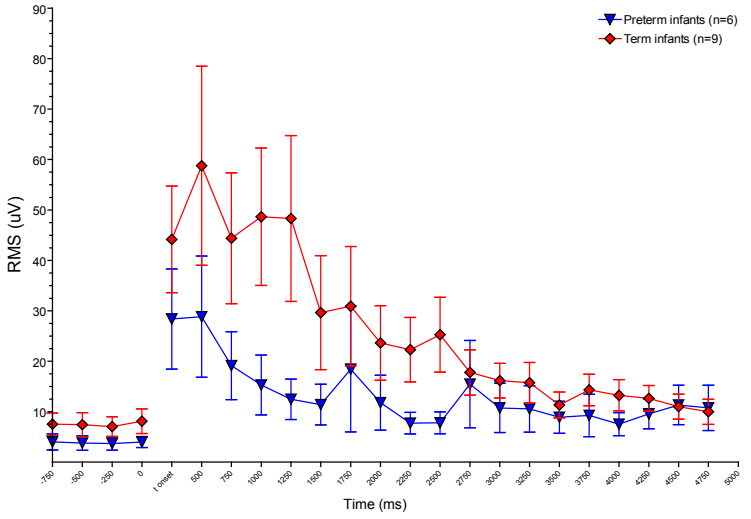


Figure 3-18: Preterm (n=6) and full-term (n=9) mean ipsilateral biceps femoris activity following non-noxious stimulation

Ipsilateral biceps femoris activity in infants with a detectable EMG reflex response. The RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recordings epoch (in completed 250ms time bins) was calculated. The RMS (±standard error) in each time bin is displayed.

3.4.9 The specificity of the flexion withdrawal reflex response to high-intensity mechanical stimulation

Flexion withdrawal reflex characteristics were compared between infants who exhibited a reflex response to a noxious heel lance, and infants who exhibited a reflex response to both noxious and non-noxious stimuli. A noxious heel lance reproducibly evoked flexion withdrawal reflex response in 100% of infants across all gestational ages. A non-noxious touch to the heel (using the lancet device) additionally evoked a reflex response in 36% of test occasions: 39% (9/23) full-term and 32% (6/19) preterm infants. These observations indicate that flexion withdrawal reflex response can be selective for a noxious stimulus, but is not specific to noxious stimuli in preterm or full-term infants.

Infants were separated into groups depending on gestational age and the specificity of flexion withdrawal reflex activity following stimulus application; full-term, n=15; preterm, n=13. The numbers included in the analysis were small due to exclusion if no non-noxious touch stimulus was performed (n=3) or technical failure in the EMG recording (n=10) i.e. no time-locked event mark or noisy baseline.

Group 1: Noxious-specific reflex responders

Where flexion withdrawal reflex activity was *only* evoked following a noxious heel lance in full-term (n=9) and preterm (n=9) infants.

Group 2: Non-specific reflex responders

Flexion withdrawal reflex activity was evoked after *both* noxious heel lance and non-noxious touch stimulation in full-term (n=6) and preterm (n=4) infants.

Firstly, the background demographics were assessed to identify if age (length of hospital stay) was a contributory factor for group allocation. Gestation and postnatal age were not significantly different between the groups (Table 3-6).

Secondly, the flexion withdrawal reflex characteristics were compared (1) between groups following a noxious heel lance, and (2) within non-specific reflex responders (Group 2) for noxious and noxious-evoked EMG activity.

	Group 1: Noxious-specific responders	Group 2: Non-specific responders	p-value
Preterm infants (n)	9	4	
Gestational age (weeks)	34.38 (33.10-35.66)	33.97 (31.19-36.74)	0.69
Post-natal age (days)	21.00 (3.45-38.55)	20.75 (-11.58-53.08)	0.99
Full-term infants (n)	9	6	
Gestational age (weeks)	39.06 (38.11-40.02)	39.14 (38.12-40.69)	0.61
Post-natal age (days)	14.44 (-8.25-37.14)	51.33 (3.36-99.30)	0.08

Table 3-6: Gestation and postnatal age are not identifying factors for infants with non-specific reflex responses
All data expressed as mean (95% CI). Statistical analysis performed using Student's unpaired t-test.

3.4.9.1 Differences in latency to onset and peak activity

(1) Onset latency

Individual onset latencies for full-term infants after a noxious heel lance [Group 1 (noxious-specific reflex responders) and Group 2 (non-specific reflex responders)] and a non-noxious touch stimulus (Group 2 only) are shown in Figure 3-19. Noxious-evoked reflex onset latency was not significantly different between Group 1, 338.6ms (95% CI 194.8-482.3ms), and Group 2, 425.3ms (95% CI 267.5-654.0ms); unpaired t-test, p=0.35. Further, no differences in onset latency between stimulus modalities in Group 2 responses [heel lance, verses non-noxious touch, 560.2ms (95% CI 292.6-827.7ms)]; paired t-test, p=0.29.

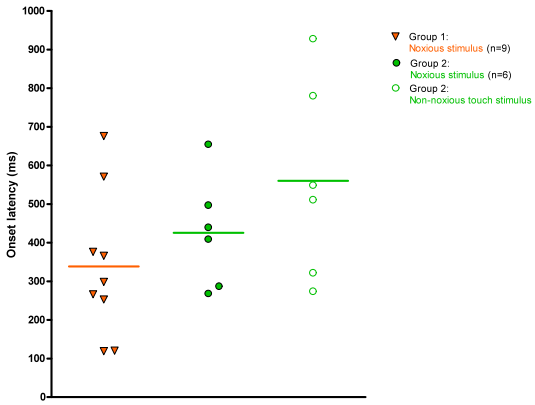


Figure 3-19: Reflex onset latency in noxious-specific reflex responders (Group 1) and non-specific reflex responders (Group 2) in full-term infants is not significantly different
Each point represents an individual infant. Onset latencies between Group 1 (n=9) and Group 2 (n=6) evoked after a noxious stimulus were not significantly different (unpaired t-test, p=0.35). In Group 2, onset latency was not significantly different between stimulus types (paired t-test, p=0.29).

For preterm infants, individual onset latencies following a noxious heel lance and a non-noxious touch stimulus are shown in Figure 3-20. Noxious-evoked reflex onset latency was not significantly different between Group 1, 486.6ms (95% CI 296.1-677.0ms) or Group 2, 212.4ms (95% CI 88.37-336.4ms); unpaired t-test, p=0.058. Nor between Group 2 stimulus modalities, [non-noxious touch, 351.8ms (95% CI -81.80-785.3ms], paired t-test, p=0.46.

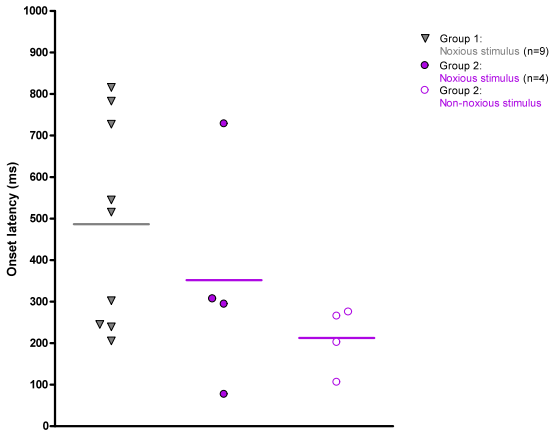


Figure 3-20: Reflex onset latency in noxious-specific reflex responders (Group 1) and non-specific reflex responders (Group 2) are not significantly different in preterm infants
Each point represents an individual infant. In Group 2, onset latency was not significantly different between stimulus types (paired t-test, p=0.46). Onset latencies between Group 1 (n=9) and Group 2 (n=4) evoked after a noxious stimulus were not significantly different (unpaired t-test, p=0.058).

(2) Peak latency

In full-term infants, peak latency was not significantly different between Group 1, 944.4ms (95% CI 496.1-1393.0ms) and Group 2, 916.7ms (95% CI 645.7-1188.0ms); unpaired t-test, p-0.92. However in Group 2, a slower build-up of motor activity occurred following a non-noxious touch as detected by a significantly longer peak latency compared to a noxious heel lance [non-noxious, 1375.0ms (95% CI 945.9-1806.0ms)]; paired t-test, p=0.04 [Figure 3-21].

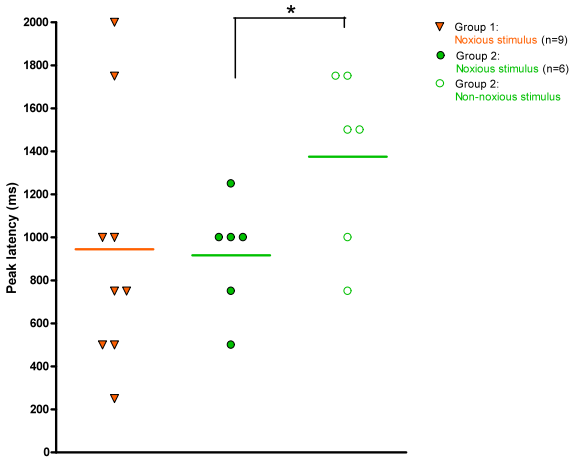


Figure 3-21: Reflex peak latency in noxious-specific reflex responders (Group 1) and non-specific reflex responders (Group 2) in full-term infants
Each point represents an individual infant. Peak latencies between Group 1 (n=9) and Group 2 (n=6) were not significantly different (unpaired t-test, p=0.92). For Group 2, peak latency was significantly shorter following a noxious stimulus compared to a non-noxious stimulus (paired t-test, p=0.038).

Preterm infants did not exhibit significant differences in latency to peak activity between Group 1, 1167.0 (95% CI 912.5-1421.0ms) or Group 2, 1500.0ms (95% CI 581.3-2419.0ms); unpaired t-test, p=0.21. Nor to stimulus types: noxious heel lance, and non-noxious touch, 1063.0ms (95% CI 16.28-2109ms) using a paired t-test, p=0.45.

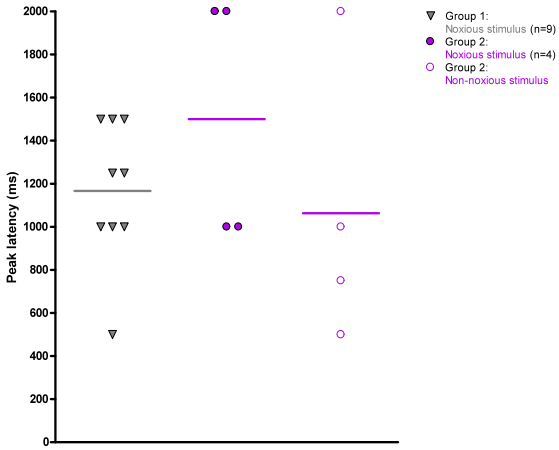


Figure 3-22: Peak latency in noxious-specific reflex responders (Group 1) and non-specific reflex responders (Group 2) in preterm infants
Each point represents an individual infant. For Group 2, peak latency was not significantly different between stimulus types (paired t-test, 0.45). Peak latencies between Group 1 (n=9) and Group 2 (n=4) evoked after a noxious stimulus were not significantly different (unpaired t-test, p=0.21).

3.4.9.2 Differences in the pattern of EMG activity

The pattern of flexion withdrawal reflex activity using latency-corrected analysis for each age group is shown on page 112. There were no differences in the pattern of activity between groups following a noxious heel lance; preterm ($F_{1,242}$=0.78, p=0.40; Figure 3-25) or full-term ($F_{1,299}$=2.05, p=0.18; Figure 3-26). Statistical testing for differences in the pattern of activity between stimulus types in Group 2 (non-specific reflex responders) also found no significant differences in activity at preterm ($F_{1,132}$=2.16, p=0.19; Figure 3-25) or full-term ($F_{1,230}$=4.30, p=0.06; Figure 3-26).

3.4.9.3 Differences in mean activity over 2000ms from onset of the reflex

Mean activity over 2000ms from the onset of the flexion withdrawal reflex response was calculated to summarise the magnitude of EMG activity.

In full-term infants, a noxious heel lance evoked significantly larger mean activity in Group 2 infants (non-specific reflex responders), 45.26μV (95% CI 26.89-63.64μV, compared to Group 1 infants, 29.61 μV (95% CI 21.60-37.62 μV); unpaired t-test, p=0.05 [Figure 3-23]. In Group 2 infants, there were no stimulus specific differences in the size of the evoked response; non-noxious, 29.11μV (95% CI -4.97-63.19μV); paired-t-test, p=0.33.

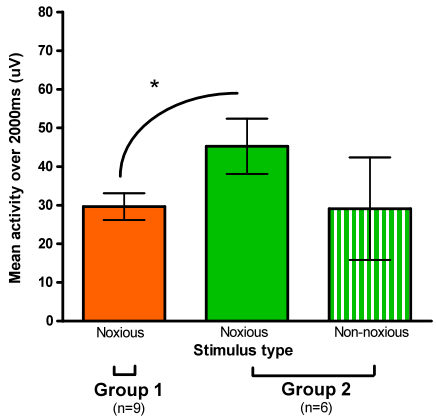


Figure 3-23: Mean activity over 2000ms is significantly greater in Group 2 infants compared to Group 1 infants following a noxious heel lance in full-term infants
Mean activity over 2000ms was calculated from the onset latency. The mean activity of the reflex response was significantly greater in Group 2 (non-specific responders) infants compared to Group 1 infants; unpaired t-test, p=0.05. There were no differences in the reflex response after a noxious and non-noxious stimulus in Group 2 infants; paired t-test, p=0.33.

In preterm infants, mean activity over 2000ms from onset was not significantly different between Groups 1 and 2 following a noxious heel lance, unpaired t-test, p=0.62 [Group 1, 42.31μV (95% CI 19.34-65.27μV); Group 2, 54.21μV (95% CI -14.79-123.2μV)]; Figure 3-24. In Group 2 infants, there were no stimulus specific differences in the size of evoked response; non-noxious, 28.30μV (95% CI 13.05-43.55μV)]; paired t-test, p=0.26.

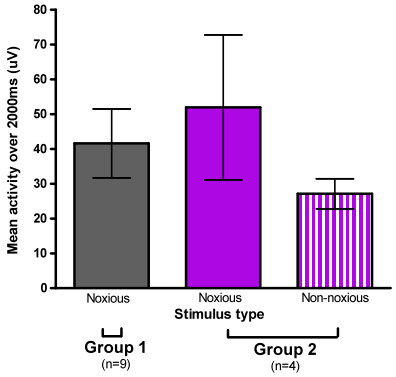


Figure 3-24: No differences in mean activity over 2000ms between groups or stimulus modalities (for Group 2) in preterm infants
Mean activity over 2000ms was calculated from the onset latency. The mean activity of the reflex response was not significantly difference between groups; unpaired t-test, p=0.62. There were no differences in the reflex response after a noxious and non-noxious stimulus in Group 2 infants; paired t-test, p=0.26.

Summary 3

- Preterm and full-term infants reproducibly exhibit flexion withdrawal reflex activity after a noxious stimulus but this response is not noxious specific.
- Infants were separated into groups depending on reflex excitability. The majority were classified as noxious-specific reflex responders. Yet, 32% preterm infants and 34% of full-term infants were non-specific reflex responders. Sensitivity to both noxious and innocuous stimuli is independent of gestation or postnatal age.
- Preterm infants did not exhibit any differences when EMG characteristics were compared (1) between groups following a noxious heel lance, or (2) within non-specific responders (Group 2) for noxious and non-noxious evoked EMG activity.
- Full-term infant non-specific responders (Group 2) took a longer time to peak activity after a non-noxious stimulus compared to a noxious heel lance. No other differences in EMG characteristics were present when stimulus responses were compared.
- Full-term infants who were non-specific responders exhibited greater noxious-evoked reflex EMG activity compared to noxious-specific responders.

Full-term: Latency-corrected

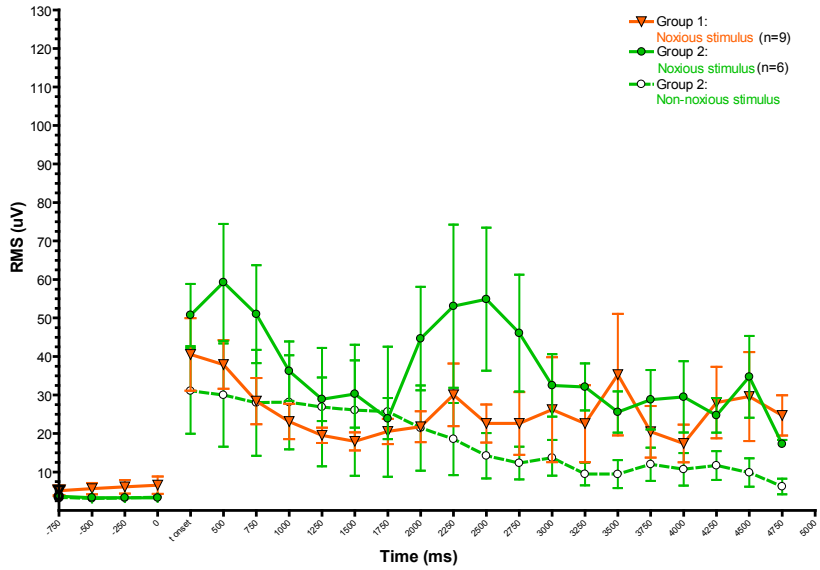


Figure 3-25: Full-term infants noxious and non-noxious evoked flexion withdrawal reflex activity
Ipsilateral biceps femoris activity was analysed in Group 1 (noxious-specific) and Group 2 (non-noxious specific) responders. The RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recordings epoch (in completed 250ms time bins) was calculated. The RMS (±standard error) in each time bin is displayed.

Preterm: Latency-corrected

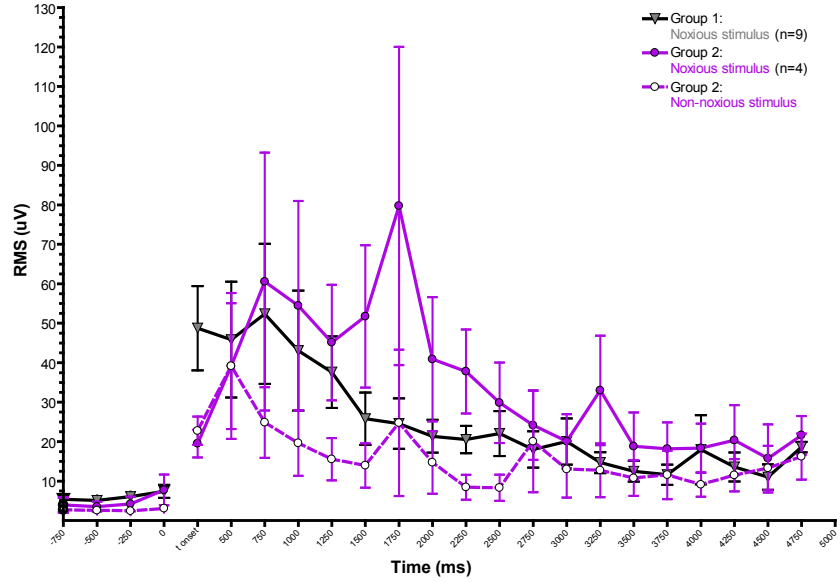


Figure 3-26: Preterm infant noxious and non-noxious evoked flexion withdrawal reflex activity
Ipsilateral biceps femoris activity was analysed in Group 1 (noxious-specific) and Group 2 (non-noxious specific) responders. The RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recordings epoch (in completed 250ms time bins) was calculated. The RMS (±standard error) in each time bin is displayed.

3.4.10 Coordination of ipsilateral and contralateral limbs following noxious and non-noxious stimulation of the heel

In this section, the laterality of flexion withdrawal reflex was investigated using paired EMG recordings of ipsilateral and contralateral biceps femoris activity. Reflex EMG activity was quantified in preterm and full-term infants after a (1) noxious heel lance and/or (2) non-noxious touch stimulation to the heel. Infant demographics of EMG recordings included in the analysis for each age group and stimulus type are in Table 3-7.

Contralateral EMG activity was initially characterised in full-term and preterm infants for each stimulus type; noxious heel lance and non-noxious touch with the lancet device. Comparisons in the flexion withdrawal reflex motor activity were then made between ipsilateral and contralateral biceps femoris.

(1) Noxious heel lance	Preterm (N=15)	Full-term (N=11)
Male; (n/N)	47%; (7/15)	55%; (6/11)
Mean GA at birth (weeks)	30.96±4.21; range 23.43-36.00	35.81±5.75; range 24.86-41.57
Mean GA at study (weeks)	34.18±1.68; range 31.43-36.29	39.44±1.44; range 37.00-42.29
Mean PNA (days)	22.53±23.94; range1-69	25.45±37.73; range 2-101
Mean weight at study (g)	1683.73±511.36; range 850.0-2780.0	2855.00±539.50; range 1959.0-3580.0
Right heel stimulated; (n/N)	40%; (6/15)	55%; (6/11)
(2) Non-noxious touch	**Preterm (N=12)**	**Ful-term (N=12)**
Male; (n/N)	58%; (7/12)	58% (7/12)
Mean GA at birth (weeks)	31.14±3.83; range 23.43-36.00	35.98±6.69; range 24.86-41.57
Mean GA at study (weeks)	33.63±2.27; range 28.57-36.29	40.07±1.56; range 38.00-42.29
Mean PNA (days)	17.33±19.56; range 1-64	28.67±45.35; range 1-116
Mean weight at study (g)	1606.67±596.68; range 850.0-2780.0	2876.17±689.63; range 1923.0-3950.0
Right heel stimulated; (n/N)	33%; (4/12)	67%; (8/12)

Table 3-7: Infant demographics for (1) noxious heel lance and (2) non-noxious touch in preterm and full-term infants
Data expressed as mean±SD unless otherwise stated.

3.4.10.1 Incidence of contralateral activity

A noxious heel lance evoked detectable flexion withdrawal reflex activity in the contralateral biceps femoris of all full-term (n=11) and all preterm infants (n=15).

Non-noxious stimulation with the lancet device evoked ipsilateral flexion withdrawal reflex activity in a small number of 5 full-term (42%) and 4 preterm (33%) infants. Of these, simultaneous contralateral activity was detected in half the sample of each age group: 2 full-term and 2 preterm infants. The small number of infants with evoked activity meant the EMG characteristics could not be comprehensively characterised further in this section. These data suggest that detectable contralateral reflex activity is less likely to occur after a non-noxious touch compared to a noxious heel lance.

3.4.10.2 Contralateral latency to onset and peak activity

A noxious heel lance

The latency to onset of flexion withdrawal reflex activity is illustrated in Figure 3-27 on page 115. In full-term infants, the mean latency to onset of contralateral flexion withdrawal reflex activity was 487.6ms (95% CI 354.5-620.7ms). In preterm infants, the latency to onset was 547.5ms (95% CI 411.0-683.9ms).

The time taken to reach peak amplitude in full-term infants was 909.1ms (95% CI 706.5-1112.0ms) and in preterm infants was 1383.0ms (95% CI 1128.0-1639.0ms).

3.4.10.3 Contralateral pattern of EMG activity

A noxious heel lance

The pattern of activity for the group average of each age group when non-latency corrected are shown in Figure 3-29 for full-term and Figure 3-30 for preterm on page 118 to illustrate the pattern of activity in real time.

In full-term infants, the corrected peak activity was 50.00μV (95% CI 30.28-69.71μV) and occurred within 250ms from the onset of flexion withdrawal reflex activity (Figure 3-31). EMG activity decreased over the following 750ms to 21.48μV (95% CI 14.33-28.64μV) and remained at a constant level until 2250-25000ms where a second burst of activity occurred.

In preterm infants, evoked activity steadily increased over the first 1000ms. Corrected peak activity was 60.40µV (95% CI 24.97-95.82µV) and occurred at 750-1000ms (Figure 3-32 on page 119). Flexion withdrawal reflex activity slowly decreased over the remainder of the recording and did not return to baseline levels.

3.4.11 Comparison of ipsilateral and contralateral flexion withdrawal activity

Ipsilateral and contralateral EMG activity was compared in preterm and full-term infants following a noxious heel lance.

3.4.11.1 Contralateral latencies are similar to ipsilateral reflex activity

Visual inspection illustrated that the onset latency was shorter in the ipsilateral than the contralateral limb (Figure 3-27). Despite this, there were no significant differences in onset latency between limbs in full-term infants [ipsilateral, 405.2ms (95% CI 284.3-256.2ms) and contralateral, 487.6ms (95% CI 354.5-620.7ms); unpaired t-test, p=0.32]. Likewise, in preterm infants, there were no significant differences [ipsilateral biceps femoris, 428.6ms (95% CI 297.8-559.5ms) and contralateral, 547.5ms (95% CI 411.0-683.9ms)]; unpaired t-test, p=0.19.

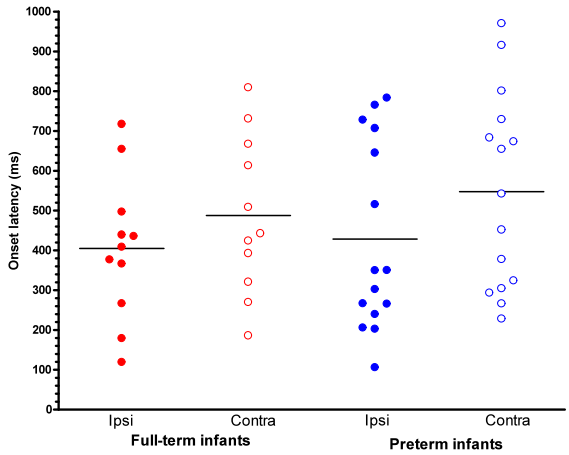


Figure 3-27: Contralateral onset latency is slightly longer in preterm and full-term infants, although not significantly different
Each point represents an individual infant. Ipsi, ipsilateral; contra, contralateral.

Peak latency was not significantly different between ipsilateral and contralateral muscles in either age group. In full-term infants, ipsilateral peak latency was 977.3ms (95 % CI 621.4-1333.0ms) and contralateral was 909.1ms (95% CI 706.5-1112.0ms); unpaired t-test, p=0.63.

For preterm infants, ipsilateral peak latency was 1130.0 (95% CI 851.0-1409.0ms) and contralateral, 1383.0ms (95% CI 1128.0-1639.0ms); unpaired t-test, p=0.16.

3.4.11.2 Ipsilateral and contralateral biceps femoris activity is similar for noxious stimulation

A clear withdrawal response was observed in ipsilateral and contralateral biceps femoris in 100% of test occasions for full-term and preterm infants. Latency-corrected activity for full-term (Figure 3-31) and preterm (Figure 3-32) is shown on page 119. The pattern of ipsilateral activity versus contralateral activity was analogous for full-term (n=11; $F_{1,396}$=0.33; p=0.57) and preterm (n=15; $F_{1,616}$=0.43; p=0.52) infants.

For noxious stimulation, the summary magnitude of activity (mean activity over 2000ms) was similar in the ipsilateral versus contralateral biceps femoris for full-term and in preterm infants (Figure 3-28). Full-term ipsilateral activity was 36.12μV (95% CI 22.85-49.39μV) versus contralateral activity, 36.59μV (95% CI 25.49-47.69μV). Ipsilateral activity in preterm infants measured 53.39μV (95% CI 35.65-71.13μV) and contralateral activity was 50.02μV (95% CI 31.66-68.37μV). There were no significant differences at any age, however there is a clear trend for ipsi- and contralateral evoked responses in preterm infants to be greater than full-term infants (Two-way ANOVA; age, $F_{1,48}$=3.95, p=0.052; side, $F_{1,48}$=0.04, p=0.85).

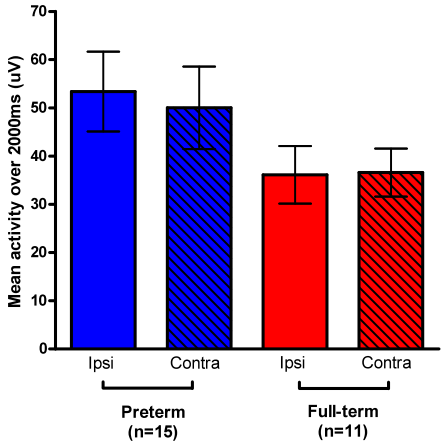


Figure 3-28: Ipsilateral and contralateral mean activity was not significantly different following a noxious heel lance
Ipsi, ipsilateral; contra, contralateral.

Summary 4

- The nociceptive flexion withdrawal reflex is a bilateral response, with synchronous ipsilateral and contralateral motor activity evoked even at full-term gestation.
- The characteristics of nociceptive reflex activity were synonymous in each muscle with no differences detected in latency measures, pattern of activity and mean activity over 2000ms and latency measures for each age group.
- Non-noxious touch stimulation of the heel with the lancet device is less likely to evoke contralateral reflex activity. Of the infants who exhibit reflex sensitivity to stimulation (42% full-term and 33% preterm), a contralateral response was detected in half the sample of each age group.
- The characteristics of non-noxious evoked contralateral EMG activity were not quantified due to small numbers (full-term, n=2; preterm, n=2).

Full-term: Non-latency corrected

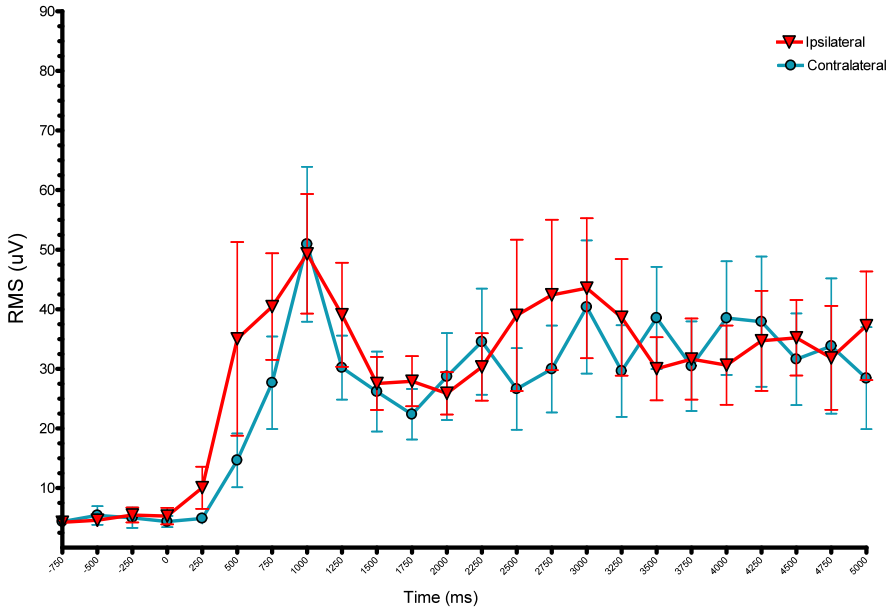


Figure 3-29: Flexion withdrawal reflex activity of the ipsilateral and contralateral biceps femoris in full-term infants following a noxious heel lance (n=11)
The RMS±(standard error) of each time bin is shown i.e. -750 = activity between -1000ms and -750ms.

Preterm: Non-latency corrected

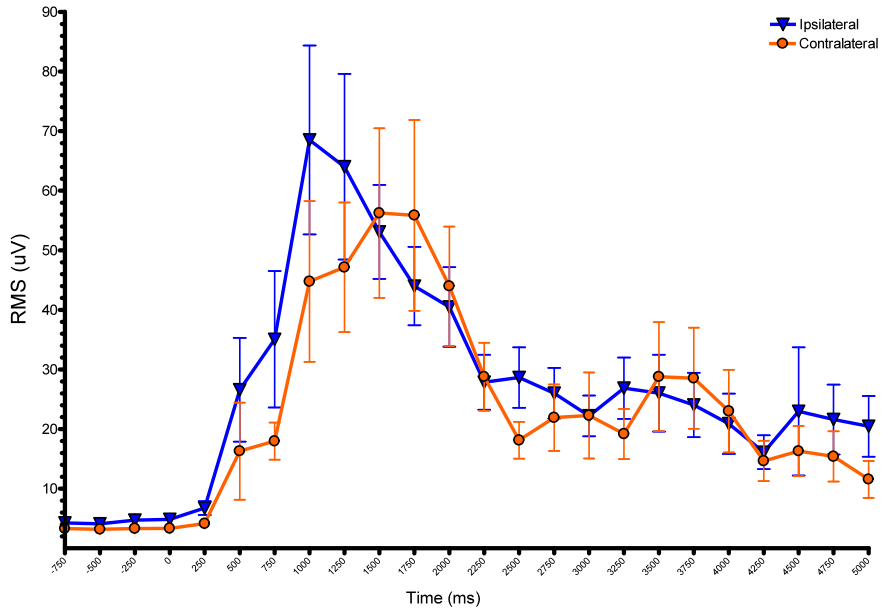


Figure 3-30: Flexion withdrawal reflex activity of the ipsilateral and contralateral biceps femoris in preterm infants following a noxious heel lance (n=15)
The RMS±(standard error) of each time bin is shown i.e. -750 = activity between -1000ms and -750ms.

Full-term: Latency-corrected

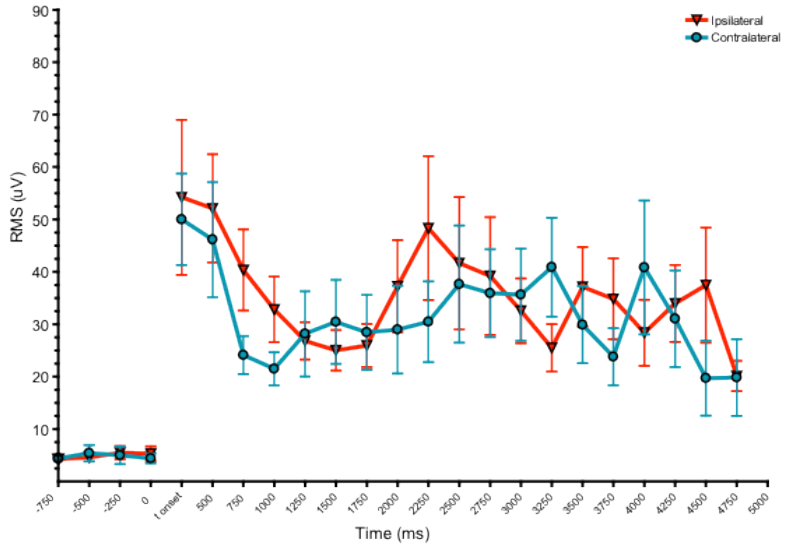


Figure 3-31: Full-term infant ipsilateral and contralateral flexion withdrawal reflex activity (n=11)
The RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recordings epoch (in completed 250ms time bins) was calculated. The RMS (±standard error) in each time bin is displayed.

Preterm: Latency-corrected

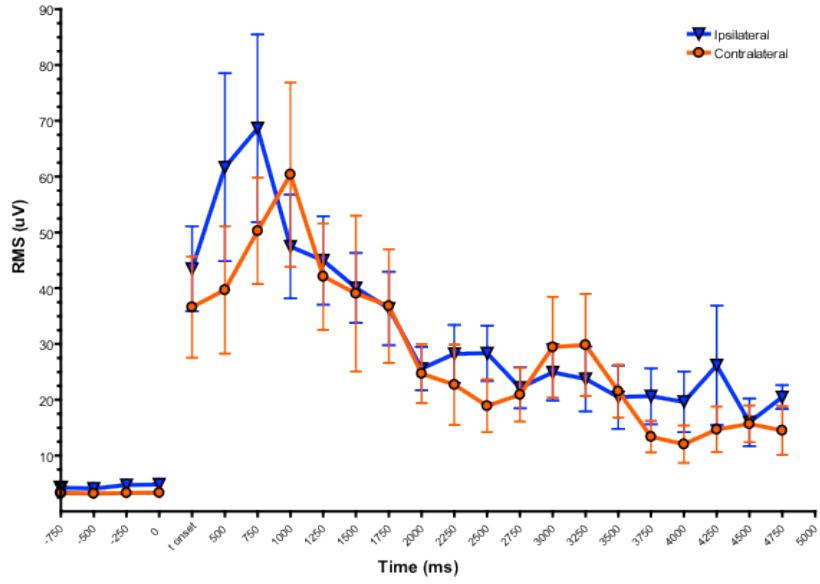


Figure 3-32: Preterm infant ipsilateral and contralateral flexion withdrawal reflex activity (n=15)
The RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recordings epoch (in completed 250ms time bins) was calculated. The RMS (±standard error) in each time bin is displayed.

3.4.12 The relationship between nociceptive flexion withdrawal reflex activity and other nocifensive behaviour: facial expression

Previous work in this chapter has demonstrated that a noxious heel lance will evoke reproducible flexion withdrawal reflex activity in infants of all gestational ages. This section investigates the relationship between nociceptive flexion withdrawal reflex activity and other pain-associated behaviour: facial expression. Ipsilateral EMG recordings, analysed in section 3.4.1, were paired with video recordings of facial behaviour following a noxious heel lance. Thirty-eight infants were included in the final analysis: 20 preterm and 18 full-term (2 infants were excluded from analysis due to technical failure in the video recording).

Noxious-evoked facial expression was first analysed (as described in section 3.3.4.2) in preterm and full-term infants. Subsequently, the relationship between facial activity and key EMG characteristics were tested for each age group.

3.4.12.1 The majority of infants exhibit facial responses to a noxious heel lance

Baseline behavioural scores were 'zero' over the 15s period prior to the heel lance. A clear facial response consisting of brow bulge, eye squeeze and/or nasolabial furrow was exhibited in 90% of preterm infants and 83% full-term infants following a noxious heel lance. There were no significant differences in the measures of facial behaviour between preterm and full-term infants [Table 3-8].

	Preterm (N=20)	Full-term (N=18)	p-value
Observed facial response (%; n/N)[1]	90% (18/20)	83% (15/18)	1.00
Latency to first observed facial response (s)[2]	3.22 (0.55-5.90)	2.80 (0.90-4.70)	0.29
Total Facial Score[3]	3.95 (2.44-5.46)	3.56 (1.99-5.12)	0.71

Table 3-8: Facial behaviour in preterm and term infants following a noxious heel lance
Data is expressed as mean (95% CI) unless otherwise stated. [1.] Fisher's exact test; [2.] Mann-Whitney t-test; [3.] Student's unpaired t-test.

3.4.12.2 The incidence of flexion withdrawal and visible changes in facial behaviour

A noxious heel lance evoked a clear flexion withdrawal reflex response in 100% of test occasions. In contrast, a visible change in facial behaviour did not occur with such high

frequency; a change in facial activity was absent in a small number of infants, 10% preterm and 17% full-term. Although a higher percentage of full-term infants exhibited a reflex response in the absence of facial behaviour, the association between age and incidence of facial response were not related (Fisher's exact-test, p=1.00, P1-P2=0.02).

3.4.12.3 Latency to peak EMG activity and first facial response is not correlated

The latency to peak EMG activity was compared against the time to first observed facial responses for 5s after the stimulus. Infants who did not exhibit a facial response within the first 5s after the stimulus were excluded from the analysis. No significant relationship between the onset of lower limb activity and facial motor activity was found in this sample of preterm infants (R^2=0.07; p=0.34) and full-term infants (R^2=0.01; p=0.70) using Pearson's Correlation. In five infants, irrespective of gestation, a facial response was absent over the first 30s after the stimulus but was not an indicator for delayed peak EMG activity.

3.4.12.4 Magnitude of flexion withdrawal reflex activity and facial response are not correlated

The magnitude of motor activity was summarised for the flexion withdrawal reflex response by calculating the RMS of EMG activity over 2000ms after the stimulus, and for the facial response in infants with a TFS>0. As Figure 3-33 illustrates, there was no relationship between the magnitude of evoked flexion withdrawal reflex activity and facial response. Correlation analysis confirmed no significant differences in preterm (R^2 =0.68; p=0.35) or full-term infants (R^2=0.005; p=0.81).

Earlier in this chapter (section 3.4.9 on page 106), infants were separated into two groups depending on their sensitivity to non-noxious stimulation. Full-term infants who were non-specific reflex responders exhibited significantly larger EMG activity (mean RMS over 2000ms) compared to noxious-specific responders following a noxious heel lance. The Total Facial Score (TFS) of these two groups were assessed for each age group to see if differences in facial behaviour were also apparent. For full-term infants, non-specific responders (n=6) evoked a similar TFS score compared to noxious-specific responders (n=8; one infant excluded due to video failure). There were no significant differences [non-specific, 3.83 (95% CI 1.59-6.08) versus noxious-specific, 3.75 (95% CI 0.24-7.26)]; unpaired t-test, p=0.97. For preterm infants, there were also no significant differences between groups [non-specific (n=4), 4.50 (95% CI -0.27-9.27) versus noxious-specific (n=8; one infant excluded due to video

failure), 3.25 (95% CI 0.43-6.07)] unpaired t-test, p=0.55. These results indicate that a large EMG reflex response does not necessarily predict that large facial behaviour will simultaneously occur in preterm and full-term infants.

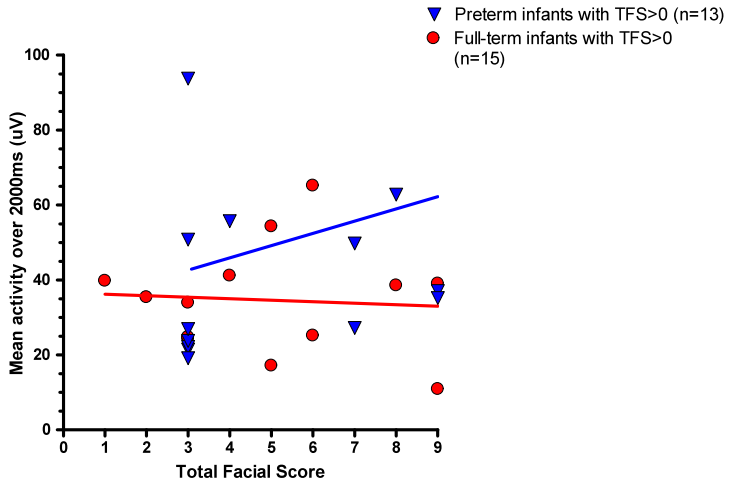


Figure 3-33: Magnitude of flexion withdrawal reflex activity and total facial score are not correlated
Correlation analysis confirmed no significant differences in preterm (R^2 =0.68; p=0.35) or full-term infants (R^2=0.005; p=0.81). Each point represents an individual infant.

Summary 5

- A noxious heel lance reproducibly evoked a flexion withdrawal reflex response in preterm and full-term infants. In most infants a change in facial behaviour occurred but was not observed with every subject.
- Using EMG and video recording techniques, these data show that there is no clear relationship between the flexion withdrawal reflex activity and facial activity when comparing the magnitude and latency characteristics of infant nociceptive motor behaviour.
- Furthermore, an infant without clear facial motor activity can still mount flexion withdrawal reflex activity that is equivalent to those infants displaying the greatest facial behaviour.

3.4.13 Summary of results

(1) Noxious heel lancing and non-noxious touch stimulation were performed in preterm and full-term infants and the associated flexion withdrawal reflex activity measured using surface EMG recordings of biceps femoris activity.

(2) All infants responded to a noxious heel lance; preterm infants took longer to mount a reflex response compared to full-term infants.

(3) Flexion withdrawal reflex activity is not always specifically elicited by a noxious stimulus. In more than 30% of test occasions, infants responded to non-noxious stimulation of the heel with flexion withdrawal reflex activity; this was independent of gestation or post-natal age.

(4) Full-term infants who were sensitive to both types of stimulation took a longer time to build up the response after a non-noxious stimulus compared to a noxious heel lance. Further, this group of infants exhibited significantly greater reflex activity following a noxious heel lance compared to infants who were noxious-specific reflex responders.

(5) The nociceptive flexion withdrawal reflex is a bilateral response, with synchronous ipsilateral and contralateral motor activity evoked even at full-term gestation. Non-noxious stimulation of the heel is less likely to evoke contralateral reflex activity.

(6) A noxious heel lance evoked a change in facial expression in most, but not all infants. There is no clear relationship between the properties of flexion withdrawal reflex EMG activity and observed facial behaviour. These results show an infant without clear change in facial expression is still capable of mounting flexion withdrawal reflex activity, further the magnitude of reflex activity is independent of TFS.

3.5 Discussion

In this chapter, direct measurement of flexion withdrawal reflex motor activity was used to provide systematic analysis of spinal cord excitability in preterm and full-term infants following noxious and non-noxious touch stimulation to the heel. Surface EMG recordings of flexion withdrawal reflex activity have been examined in the neonate previously but have not unravelled the developmental morphology or provided comprehensive characterisation of muscle activity (Andrews *et al.*, 2000; Andrews *et al.*, 1994).

The initial aim of this chapter was to examine the EMG characteristics of noxious-evoked flexion withdrawal reflex activity, and to use this information to investigate whether developmental changes were apparent; the present results provide detailed characterisation of the flexion withdrawal reflex properties across a range of gestational ages and are novel in presenting age-associated comparisons. A further aim was to investigate the stimulus specificity of the reflex by comparing noxious-evoked activity against non-noxious touch associated activity; the present results confirm previous findings and show that reflex activity can be evoked by a low-intensity innocuous stimulus in some infants (Andrews *et al.*, 1999). Coordination of limb movement was investigated by analysing EMG activity from the ipsilateral and contralateral biceps femoris following a noxious and non-noxious stimulation; the present results show for the first time in the human infant that reflex withdrawal consists of activation of both biceps femoris, and that this is affected by the intensity of stimulation – being more apparent with high-intensity noxious stimulation. Finally, the relationship between facial activity and limb withdrawal was investigated; these findings showed that whilst flexion withdrawal could always be evoked after a heel lance, facial activity was not always observed. Furthermore, there was no clear relationship between the properties of the flexion withdrawal EMG activity and visual observations of facial motor activity.

3.5.1 Stimulus/response characteristics of flexion withdrawal reflex activity

3.5.1.1 Incidence

The present results show that a noxious heel lance evoked flexion withdrawal reflex activity in every infant. The spinal reflex arc is fully functional and reliable even in the youngest infants who were studied at 30 weeks GA. *In utero* reflex studies show cutaneous reflex activity is detectable between E15-E19 days in the rat and 10-12 GA weeks in the human. Postnatal

studies examining reflex responses in the kitten (Ekholm, 1967) and rat (Fitzgerald *et al.*, 1984; Fitzgerald *et al.*, 1988b) are in agreement with the present results and demonstrate cutaneous sensitivity from birth. Further, lower limb withdrawal in the human infant following natural innocuous stimulation have used behavioural measures to show that flexion withdrawal is observed in infants as young as 28 weeks GA (Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b).

The stimulus/response characteristics of flexion withdrawal following a noxious heel lance were analysed using surface EMG recordings of ipsilateral biceps femoris activity for preterm and full-term infants. Extensive information could be drawn from each EMG recording and provided an objective, quantitative method to analyse motor activity; for each infant the latency to reflex activity and peak activity, along with the pattern of activity were determined. These data provide baseline characteristics of flexion withdrawal reflex activity in the neonate for use in subsequent chapters in this thesis investigating the effect of increasing stimulus intensity, repeated stimulation and modulation of spinal reflex activity with oral sucrose. The present results show that in all infants, flexion withdrawal was characterised by a rapid onset of activity that was maintained above baseline levels for the duration of the epoch recording.

3.5.1.2 Onset and peak latency

In comparison to adults, latency to onset of reflex activity was relatively long in preterm and full-term infants (404.4ms and 534.6 ms respectively). This was in agreement with previous studies investigating limb withdrawal to noxious heel lance in the neonate (302.0 ms; Andrews *et al.*, 1999). By comparison, normative values in the adult following noxious electrical stimulation are 85-125ms (RIII component) (Hugon, 1973). The present results showed that younger infants took longer to mount a reflex response, as reflected by the delayed onset of peak activity compared to full-term infants. Developmental delays in conduction velocities of peripheral and central components of both motor and somatosensory pathways that are known to rapidly decrease over the first 2 years of life before remaining at constant adult values thereafter (Eyre *et al.*, 1991). The reduction in latency with gestational age is consistent with the marked drop in latency of cutaneous upper limb reflexes observed between infants of 33 weeks and full-term (Issler *et al.*, 1983). While the lack of myelination and lower conduction velocities contribute to low speed of CNS processing in infants, a major factor is the prolonged central synaptic delays in the immature CNS (Fitzgerald *et al.*, 1987). Developmental switches of receptor subunits at postsynaptic sites speed up the synaptic current decay contributing to faster rising EPSPs (excitatory post synaptic potentials) and

reduced spike jitter, thereby increasing the temporal precision of synaptic transmission (Takahashi, 2005).

3.5.1.3 Pattern of activity

The pattern of flexion withdrawal reflex EMG activity has previously been characterised in adults, following electrical stimulation of sensory nerves as a biphasic response consisting of a tactile (RII) component at 56-65 ms and a later, nociceptive (RIII) component (Sandrini *et al.*, 2005). In the present study the much longer latencies of infant EMG activity and the less synchronised natural skin stimulation (as opposed to the more classical nerve stimulation) did not reveal clear RII and RIII components. RII components, are, anyway not a reproducible measure in the adult and not present in every study (Hugon, 1973; Willer, 1977). The larger time-bins of 250ms used to summarise evoked EMG activity over time may also have masked the distinction between components. This method was appropriate to use for the current dataset, as evoked activity remained raised above baseline levels for many seconds. A confound of splitting data into time-bins is that temporal resolution of the recording declines; if the time-bin size was too large, important changes in the pattern of activity would be lost, whilst if it was too small then variability would increase.

The main difference between the two ages was in the time to peak activity, which was significantly longer in preterm infants [1214.0ms (95% CI 1037.0-1392ms)] compared to full-term infants [934.2ms (95% CI 742.2-1126.0ms)]. Since, leg movement is most likely to occur at peak EMG activity, this data shows that preterm infants may move one second later than a full-term infant, following a heel lance, which is a meaningful, functional time difference and might influence clinical assessments. This is likely to be due to slower and less synchronised depolarisation and excitation of muscle in preterm infant due to immaturity of the neuromuscular junction and the contractile properties of the muscle fibres (Cook, 1981).

For other parameters, there were no significant age-dependent differences in flexion withdrawal reflex EMG activity between preterm and full-term infants. However, there is a strong trend for the preterm ipsi- and contralateral noxious evoked response to be greater overall than the full term response. Previous studies show cutaneous reflex EMG patterns of responses develop over time during childhood; between birth and the first 2-3 years of age are an important period for maturation as all the elements of an adult response are recognisable at the end of this time period, and become more adult-like with increasing age (Issler *et al.*, 1983; Mayer *et al.*, 1969; Rowlandson *et al.*, 1985). Separating of groups of preterm and full-

term infants into smaller age bands may have revealed more significant differences in reflex activity in the youngest and oldest infants, but much larger numbers of infants would be required over each age group due to variability in the response.

3.5.1.4 Reflex variability

Visual inspection of the EMG recordings indicated that in the present data more variation in the pattern of reflex EMG response was prevalent in the younger, preterm infants, with some showing an increased peak activity and prolonged response. Surface EMG recordings detect the electrical activity generated by a muscle through the overlying skin and subcutaneous tissue. The resulting signal is the result of cutaneous afferent input, dorsal horn sensory integration, activity generated in pools of motor neurons innervating the muscle, neuromuscular transmission and activity in muscle fibres. The signal morphology is dependent on the size of motor unit, frequency of motor unit discharge, and coordination of motor unit firing as well as the properties of the muscle fibres. At high levels of motor activity including flexion withdrawal, superimposed activity of multiple motor units give rise to a complicated EMG response in which individual motor units cannot be distinguished. Active motor units increase their rate of firing and new (previously inactive) motor units are also recruited. Variability of motor responses and reflex behaviour is common to the developing nervous system. Onset latencies and coordination of muscle activity are most variable in the youngest rat pups (Holmberg *et al.*, 1996). Latencies of response to A fibre skin stimulation in rat pup dorsal horn are not only very long but also vary widely in the youngest animals (Jennings *et al.*, 1998). As previously mentioned, longer conduction velocities in younger infant cause scatter or desynchronisation in the pattern of EMG activity due to the delay in reaching the muscle. The increased variation is also likely to be a result of immature synaptic kinetics and connectivity, and the fine-tuning of motor unit firing properties (Takahashi, 2005). In young animals and humans, muscle fibres are innervated by multiple rather than one motor neuron, unlike the adult, and may also contribute to increased variability (Buffelli *et al.*, 2004).

3.5.2 The effect of noxious versus non-noxious stimulation

The results show that more than 30% of infants respond to low-intensity non-noxious touch stimulation in addition to a noxious heel lance. For these infants, the flexion withdrawal reflex is therefore not stimulus-specific. These results are in agreement with cutaneous sensitivity studies that show neonatal animals (Fitzgerald *et al.*, 1988b) and human infants

(Abdulkader *et al.*, 2008a; Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b) exhibit lower sensory thresholds to stimulation that gradually increase with postnatal age. In the adult, the threshold for reflex withdrawal is much higher for both the rat (Woolf, 1984) and human (Hugon, 1973), and is associated with pain perception (Willer, 1977). The human neonate electrical flexion withdrawal reflex thresholds is closer to the threshold for the tactile flexion reflex (RII) in the adult, which suggests the involvement of Aß fibres (Andrews *et al.*, 1999). In the adult, Aß fibres are non-nociceptive but it is possible that in the neonate Aß mediated cutaneous afferents converge onto nociceptive circuitry and are able to evoke flexion withdrawal reflex activity earlier in development, and that this gradually becomes more suppressed as shown by lower reflex thresholds. Anatomical studies show the central terminals of large diameter Aß afferents that are normally located in the deeper dorsal horn can be found in the substantial gelatinosa in the neonate, and remain for up to 3 postnatal weeks in the rat (Fitzgerald *et al.*, 1994; Granmo *et al.*, 2008). Electron microscopy studies have shown that A-fibres form synaptic contacts with cells in the substantia gelatinosa before withdrawing to laminae III-V for final termination (Coggeshall *et al.*, 1996). Organisation of synaptic connections in the dorsal horn undergo significant reorganisation over the postnatal period since the immature nervous system is less selective in distinguishing low-intensity stimuli from noxious input. It is possible that superficially located Aß fibres activate dorsal horn neurons that are normally involved in the nociceptive reflex pathway. The changes in sensitivity are likely to be associated with reorganisation of central connections, specifically with regard to Aß-fibre arrangement.

Reflex responses to non-noxious touch stimulation were independent of gestational age. It can be argued that the hypothesis of enhanced Aß fibre activation (which is under developmental regulation) does not therefore hold true. Previous work has shown that infants exposed to more painful and stressful proceedures exhibit heightened responses to subsequent stimulation (Holsti *et al.*, 2005; Holsti *et al.*, 2006). It is possible that infants with increased sensitivity to non-noxious stimulation experienced recent invasive procedure(s) prior to the study, thus exhibiting nocifensive behaviour. Peripheral sensitisation (primary hyperalgesia) leads to central sensitisation as the region of skin around the affected area increases in sensitivity (Latremoliere *et al.*, 2009). Hyperalgesia following repeated heel lancing has been demonstrated in the infant (Fitzgerald *et al.*, 1988a; Fitzgerald *et al.*, 1989) and complementary studies in the neonatal rat pup show hind-paw incision lowers sensory threshold and enhances the magnitude of reflex response 24hours post-incision (Walker *et al.*, 2009b). In the current study, it was not possible to obtain the time of the last invasive

procedure, such as heel lance, however, when conducting the study the research nurse chose a stimulation site that was unlikely to be a sensitive region i.e. away from a previous lance injury.

To test whether those infants that displayed a response to innocuous stimulation were generally in a different state of excitability to those that did not, we tested the size of the noxious evoked activity in the two groups. Our data shows that in full-term infants, there is a significantly higher response to noxious stimulation in those infants that also responded to touch. This suggested that these infants were indeed sensitized, perhaps due to a previous 'pain history'. On the other hand, in preterm infants reflex activity to noxious stimulation was the same whether they were sensitive to touch or not, and furthermore the reflex responses to touch and heel lance were equivalent in magnitude (RMS over 2000ms). This suggests that the failure of the flexion reflex in preterm babies to discriminate between non-noxious and noxious stimulation is independent of the state of excitability and is therefore likely to be intrinsic to the immature reflex circuitry.

An alternative possibility is that these infants were in a hyper-alert status and flexion withdrawal was simply a salient response to the sound of the 'click' as the blade was released in the lancet device, but this is unlikely in view of the von Frey hair data presented in Chapter 4 . In adult studies, diversion of attention away from a stimulus depresses flexion withdrawal reflex activity and likewise, attending to a stimulus facilitates flexion withdrawal activity (Sandrini *et al.*, 2005). This requires the involvement of supraspinal modulation over the spinal reflex circuitry, and ultimately a degree of awareness of the stimulus. With regard to the former, supraspinal centers are important in maintaining the integrity and proper excitability of adult spinal reflex circuits, but also for functionally tuning these circuits during postnatal development (Levinsson *et al.*, 1999a). Brainstem circuitry is immature before the 3rd post-natal week in the rat (Fitzgerald *et al.*, 1986; Hathway *et al.*, 2009).

3.5.3 Coordination of ipsilateral and contralateral reflex activity

The present results show that cutaneous evoked flexion reflex activity is evoked in both ipsilateral and contralateral limbs. Therefore, unlike the adult, the nociceptive withdrawal reflex response is a bilateral response, with synchronous ipsilateral and contralateral motor activity evoked even at full-term gestation. Reflex coordination does not mimic the high degree of localisation found in adult humans (Hagbarth, 1960). These studies are in agreement with previous studies in the kitten (Ekholm, 1967), rat-pup (Holmberg *et al.*, 1996;

Waldenstrom *et al.*, 2003) and human infant (Andrews *et al.*, 2002a; Andrews *et al.*, 2002b; Franck, 1986) which report uncoordinated gross movement of all limbs in response to a threshold stimulus.

Abnormal reflex radiation (the spread of recruited muscle other than the stimulated/stimulus-associated muscle), is extensive from birth and subject to development. Muscle coordination in response to stretch, a monosynaptic spinally coordinated reflex, consists of a radial response along the muscles of the upper and lower limbs at birth to even include antagonistic muscles, and progressively becomes more refined over 2-4 years of age (Leonard *et al.*, 1995; Myklebust *et al.*, 1993; O'Sullivan *et al.*, 1991). The gradual fine-tuning of the underlying reflex network is reflected by progressive refinement of reflex behaviour in the first three postnatal weeks in the rat (Holmberg *et al.*, 1996; Waldenstrom *et al.*, 2003). This is due to the maturation of the underlying reflex circuitry and the synaptic elimination between the efferent neurons and their target muscle. Maturation of this circuitry must carry on beyond the postnatal period and into infancy before unilateral leg flexion occurs in the human.

3.5.4 The relationship between flexion reflex and facial expression

This is the first study to examine spinal reflex EMG responses in the human infant in combination with the simultaneous changes in facial expression (using a validated clinical pain assessment scale). The present results show that a clear facial response to a noxious stimulus was observed in most, but not all subjects. There was no clear relationship between the properties of the flexion withdrawal reflex EMG activity and observed facial behaviour. An infant without clear change in facial expression is still capable of mounting flexion withdrawal reflex activity; subsequently the magnitude of reflex activity is independent of TFS score.

Following a noxious heel lance, most infants expressed a combination of facial changes including brow bulge, eye squeeze and nasolabial furrow, together with flexion withdrawal reflex activity. Facial expression is the most consistent pain-associated in the neonate compared to other behavioural and physiological measures (Grunau *et al.*, 1987). These authors report that following a noxious heel lance 96% of the infants showed brow action, 96% eye squeeze, 97% naso-labial furrow. Thus, changes in facial expression are not 100% reliably reproduced. Indeed, more recent work has also show changes in facial expression are not evoked in 30% of infants (Slater *et al.*, 2008; Slater *et al.*, 2009). Flexion withdrawal reflex activity can be evoked at lower thresholds compared to observation of facial grimacing

(Abdulkader *et al.*, 2008a). The higher incidence of flexion withdrawal reflex activity may reflect the hyperexcitability of the spinal cord relative to other components of the nervous system in the infant.

Development of the nervous system takes longer to mature at higher levels than at the spinal cord (Fitzgerald *et al.*, 1986; Hathway *et al.*, 2009; van Praag *et al.*, 1991). It is likely that the differences in evoked facial activity are due to the immaturity and complexities of the neuronal circuits involved; flexion withdrawal reflex is a relatively simple polysynaptic spinal reflex whilst facial motor activity is coordinated at the brainstem and requires activation of large pools of motor neurons to generate coordinated facial expression.

It is important to mention that EMG recordings of muscle activity are a more sensitive measure of motor changes than visual observations. Small stimulus-associated changes in facial musculature may occur that are too discrete to be detected by the naked eye. Computerised methods of quantifying facial expression changes have been proposed for the neonate and adult, where anatomical features are marked and their changes plotted over time (Lucey *et al.*, 2010; Schiavenato *et al.*, 2007). This work still maintains a level of subjectivity that EMG recordings do not have, and dependence on the infant face remaining in the same video plane.

3.5.5 Implications

3.5.5.1 Clinical practice

Clinical pain assessment tools use facial expression as an indicator of pain perception in the neonate. The difficulty of pain assessment is the reproducibility of nociceptive behaviour. Whilst a change in facial expression is reliably observed, it does not always occur with every noxious event or in each infant. In contrast, the present results show flexion withdrawal reflex activity is reproducibly evoked in the infant. In this sense it could be argued that the flexion withdrawal reflex is a more sensitive measure of nociceptive processing. However, these results also demonstrate an equivalent response occurs in some infants following innocuous stimulation to the heel, so this measure should not be used as a sole index of nociception in young infants. In addition, the flexion reflex is a measure of spinal cord nociceptive processing and facial expression involves brainstem circuits and possibly some limbic system involvement.

3.5.5.2 Research tool

This chapter has used surface EMG recordings as a method to directly measure the effect of noxious and non-noxious touch stimulation of the heel on flexion withdrawal reflex activity in preterm and full-term infants. It has demonstrated aspects of the flexion withdrawal reflex that are developmentally regulated over the preterm to full-term time period and aspects that are not. It has provided new insights into the development of human infant spinal touch and pain processing.

The techniques that have been optimised and refined here can now be used a tool for investigating the effect of repeated clinical procedures and for pharmacological and non-pharmacological agents on upon nociceptive responses in the neonate. The data in this chapter provides baseline measures to enable changes in flexion withdrawal reflex activity to be more accurately determined by assessing the degree of stimulus/response shift in individuals or groups of infants.

3.5.6 Conclusion

The results have established that all infants exhibit flexion withdrawal reflex activity to a noxious heel lance, consisting of flexion in ipsilateral and contralateral limbs. More than 30% of infants exhibit low cutaneous sensitivity and respond to non-noxious stimulation of the heel with flexion withdrawal and may be due to changes in central and peripheral nociceptive circuitry. Finally, facial pain behaviour is not associated with flexion withdrawal reflex activity.

Chapter 4

Study 2

Cutaneous sensory thresholds and the associated flexion withdrawal reflex activity following single & repeated mechanical stimulation

4 Study 2

4.1 Introduction

4.1.1 Heightened sensitivity to innocuous stimulation

In the adult, cutaneous sensitivity, as indicated by flexion withdrawal reflex activity, is specific to high-threshold noxious stimuli and associated with the threshold for pain perception (Willer, 1977). Neonatal animal and human infant studies show cutaneous sensitivity is far more sensitive than in the adult, and is responsive to low-intensity, innocuous stimulation. Fitzgerald and coworkers demonstrated that cutaneous sensory thresholds increase over development in both the neonate rat and human infant using flexion withdrawal reflex activity as a measure of sensory processing (Fitzgerald *et al.*, 1988b). Further work in humans has supported these results and collectively indicates that cutaneous sensitivity significantly lower in infants less than 37 weeks GA i.e. preterm age, and gradually rises with age (Andrews *et al.*, 1999; Andrews *et al.*, 1994); for instance, the force required to evoke limb withdrawal at 30-35 weeks GA is 0.52 g and increases at 39-44 weeks GA to 1.7g (Andrews, 1997). Spinal cord activity is more excitable in the neonate and it is clear even innocuous levels of intensity are capable of activating the neuronal circuitry underlying nociception. The gradual decrease in cutaneous sensitivity seen with age is reflective of the maturation of central processes including reorganisation of synaptic connections (Fitzgerald *et al.*, 1999).

4.1.2 Sensory input is correlated with flexion withdrawal reflex activity

The intensity of sensory input is well correlated with cutaneous flexor withdrawal reflex activity (Woolf *et al.*, 1985). Flexion withdrawal reflex properties including shorter onset latencies and larger reflex activity occur with increasing stimulus intensity in the human infant (Andrews *et al.*, 1999) and adult (Campbell *et al.*, 1991; Dimitrijević *et al.*, 1970). Noxious stimuli produce a larger flexion withdrawal reflex response than non-noxious stimuli in both the neonate (Andrews *et al.*, 1999) and adult (Willer, 1977). Additionally increased stimulus intensity evokes more globalised body movements in the neonate (Abdulkader *et al.*, 2008a).

Thus indicating the usefulness of the flexion withdrawal reflex and the relative quantitative measurement in noxious and non-noxious evoked responses in the neonate.

4.1.3 Plasticity of the central nervous system

A plastic nervous system is important for the modification of strength and effectiveness of neuronal connections by an internal or external input; plasticity forms the basis of learning and memory. Of particular importance in nociceptive processing is the ability to detect and 'remember' danger. In the adult, a tissue-damaging insult leads to prolonged post-stimulus sensory alterations such as continuing pain, and increased sensitivity to subsequent noxious and innocuous stimuli. This is characterized by a reduction in cutaneous sensory thresholds, expansion of reflex receptive fields and exaggerated reflex withdrawal activity. A combination of local changes around the site of injury and central components of the sensory processing circuitry is contributory (Julius *et al.*, 2001; Latremoliere *et al.*, 2009). The neonatal nervous system is analogous to the adult in this sense; it is clear that even preterm infants demonstrate hyperalgesia to repeated heel lancing as shown by heightened flexion withdrawal responses (Fitzgerald *et al.*, 1988a; Fitzgerald *et al.*, 1989), facial behaviour (Taddio *et al.*, 2002) and clinical pain assessment scores (Taddio *et al.*, 2009a).

Painful procedures such as heel lancing are not the only cutaneous sensory input regularly experienced by infants under medical care that lead to changes in overall sensitivity. Non-noxious routine care giving tasks such as repositioning, nappy changing and feeding occur frequently throughout the day. Clinicians try to cluster such necessary tasks together to allow infants longer periods to rest. Evidence suggests that prolonged rest periods enhance infant sleeping duration, weight gain and reduction in the incidence of apnea (Holditch-Davis *et al.*, 1995; Symanski *et al.*, 2002; Torres *et al.*, 1997). However, conflicting studies have found that excessive handling leads to greater physiological and behavioural reactivity to subsequent painful procedures including a heel lance and lumbar puncture (Porter *et al.*, 1991; Porter *et al.*, 1998). The youngest infants, less than 30 weeks GA, have been shown to find the short period of repeated stimulation to be very stressful and are more reactive when clustered care precedes blood collection as demonstrated by exaggerated facial responses and increased basal cortisol levels (Holsti *et al.*, 2005; Holsti *et al.*, 2006; Holsti *et al.*, 2007). These data suggest that a period of innocuous repeated stimuli induce changes in neonatal circuitry that is thought to be age dependent and reflective of increased excitability at the level of the spinal cord.

Repeated stimulation in the human neonate using experimentally controlled studies have shown that innocuous intensities alters spinal excitability in an age dependent manner (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). Preterm infants (<37 weeks GA) sensitise to further stimulation as shown by the increased force of withdrawal activity with rhythmic flexor and extensor movement and number of flexion withdrawal responses (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). In contrast, full-term infants (≥ 37 weeks GA) habituate to subsequent stimuli as shown by dampened reflex activity such as reduction or complete absence of leg movement (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). These data suggest that spinal cord activity is particularly plastic during development. A better understanding of the consequences of excessive handling and pain producing procedures undergone by infants needing intensive care is warranted to improve medical care in this vulnerable population.

Collectively, sensory threshold measurement and the associated flexion withdrawal reflex activity provide an indication of the levels of spinal cord excitability in the neonate. Increased basal excitability in the neonatal spinal cord as shown by lower cutaneous sensory thresholds, and the effect of repeated stimulation has been investigated previously using calibrated von Frey hairs. These studies assessed the development of reflex excitability using descriptive accounts and the number of observed limb withdrawals but do not provide quantitative measurement of cutaneous sensitivity changes in combination with flexion withdrawal reflex activity. Since many studies have depended upon behavioural observations that are qualitative and subjective in nature, here we use EMG recordings of lower limb activity to provide accurate characterisation of reflex properties in the neonate following single and repeated cutaneous stimulation.

4.1.4 Clinical Perspective

Neonates undergo many clinical procedures each day as part of their essential medical care. Clinical procedures are performed as a single one-off event or may be clustered together depending on the neonatal management strategies under use by the institution and the stability of the infant. An epidemiological study of 14 neonatal units in France found that an infant in neonatal care experienced an average of 16 painful or stressful procedures each day, which increased to 62 procedures in the sickest infants (Carbajal *et al.*, 2008). The researchers defined stressful procedures as those that caused physical uneasiness or disturbed the equilibrium of the neonate in its environment; these include physical infant handling such as repositioning, and intravenous line removal. Meanwhile, painful procedures are invasive and

range from a tissue-damaging heel lance and lumbar puncture, to nasal suctioning and cannulation. Considering that the average length of hospital stay is 56 days (spanning between 4 to 140 days) (Green *et al.*, 2005), the importance of understanding the cumulative effects of pain and stress on the developing nervous system is imperative for improving neonatal clinical care.

4.2 Aim of the chapter

The aim of this chapter was to study the postnatal development of the flexion withdrawal reflex response to single and repeated mechanical stimulation of the lower limb. Surface EMG recordings were used as a quantitative measure of flexion withdrawal reflex activity. The key objectives were:

1) To investigate cutaneous sensory threshold changes with gestational age using behavioural observation of limb movement following a mechanical stimulus
2) To examine the characteristics of surface EMG recordings as a measure of flexion withdrawal reflex activity in relation to visual observation of limb movement in preterm and full-term infants
3) To test changes in flexion withdrawal reflex excitability following a series of repeated stimuli at suprathreshold intensities using cutaneous sensory threshold and quantitative EMG measurement in the preterm and full-term infant

4.3 Methods

4.3.1 Participants

All the participants were in-patients on the NICU, SCBU, TC and Post-natal ward. The participant criteria are described in the General Methods (section 3.3.1). Twenty studies were conducted in infants aged 30 to 43 weeks GA. Infants were only studied once. Individual infant characteristics are described in Table 4-1.

Infant no.	Sex	GA_{birth} (weeks)	$Weight_{birth}$ (g)	GA_{study} (weeks)	PNA (days)	$Weight_{study}$ (g)	Reason for admission
1	Female	28.43	872	30.14	12	972	Prematurity
2	Male	28.43	1230	30.14	12	1126	Prematurity
3	Male	24.14	605	31.57	52	777	Extreme prematurity
4	Female	24.14	455	31.57	52	732	Extreme prematurity
5	Female	32.00	1914	33.14	8	1250	Prematurity
6	Female	25.57	877	33.14	53	2040	Extreme prematurity
7	Male	31.00	1737	33.43	17	1879	Prematurity
8	Male	31.29	1940	35.57	30	2700	Prematurity
9	Male	31.29	1500	35.57	30	2331	Prematurity
10	Male	32.57	1909	35.71	22	2256	Prematurity
11	Male	35.14	2737	36.00	6	2547	Prematurity
12	Male	35.14	2758	36.00	6	2614	Prematurity
13	Female	26.71	950	37.29	74	2010	Extreme prematurity
14	Female	26.86	908	37.43	74	2152	Extreme prematurity
15	Male	30.43	1440	38.14	54	2092	Prematurity
16	Female	30.43	1480	38.14	54	2110	Prematurity
17	Female	38.14	2860	38.71	4	2706	Jaundice
18	Female	38.14	2000	38.86	5	2594	Jaundice, infection
19	Female	38.14	2760	38.86	5	1920	Establishing feeds
20	Male	40.86	3440	42.29	10	3564	Infection

Table 4-1: Individual characteristics of infants included in the study
GA, gestational age; PNA, postnatal age.

4.3.2 Study design

Sensory threshold (T) was evaluated using von Frey hairs (vFhs) application to the medial plantar surface of the foot as described in the General Methods (see section 2.4.3). The calibrated values for the set of vFhs used in this chapter are listed in Table 2-1 in section 2.4.3. Sensory threshold testing was performed at the beginning of the recording to establish

control values ($\mathbf{T_c}$), and then immediately following a series of repeated stimuli, to establish the new poststimulus threshold ($\mathbf{T_{rs}}$). Spinal flexion withdrawal reflex activity and behavioural limb movement evoked at threshold intensities were measured using manual event-marking of surface EMG recordings and visual observation of limb withdrawal. A schematic diagram of the experimental time-line is shown in Figure 4-1. A detailed description of the study protocol is described below:

(1) Evaluating control sensory threshold (T_c)

VFhs were applied in ascending order of intensity until a clear, brisk withdrawal of the limb was observed. Each stimulus application was performed when the infant was resting i.e. no limb movement, and the EMG recording was stable. After each study, the EMG recording was analysed for quantification of flexion withdrawal reflex activity at behavioural sensory threshold ($\mathbf{T_c}$) and at subthreshold forces, one vFh grade below threshold ($\mathbf{T_c-1}$) and two vFh grades below threshold ($\mathbf{T_c-2}$).

Traditionally, sensory threshold is determined using the 'staircase' method where stimuli are applied at increasing and decreasing intensities until the lowest force capable of evoking a reproducible response is found. For the purposes of this study, an initial force 2 grades below the expected sensory threshold was chosen to reduce the total number of stimuli applied to the foot; avoiding habituation or sensitisation to additional stimulation. The mechanical force initially applied was 2 grades below the expected sensory threshold. Force selection was based on similar work by Andrews (1999) who also evaluated sensory threshold in the human infant using mechanical stimulation (vFhs) in a range of gestational ages.

(2) Repetitive stimulation at suprathreshold and re-evaluation of sensory threshold (T_{rs})

A single supratheshold vFh, two grades above control threshold ($\mathbf{2+T_c}$), was applied to the medial plantar surface of the foot, at the same position, at a rate of 1/10s for 2 minutes according to the method of Andrews (1999). Sensory threshold immediately following repeated stimulation was re-evaluated using the method described in (1). The initial force applied was 2 grades below the control sensory threshold ($\mathbf{T_c-2}$), followed by $\mathbf{T_c-1}$, $\mathbf{T_c}$ and $\mathbf{T_c+1}$ etc to establish the new post stimulus train threshold, $\mathbf{T_{rs}}$.

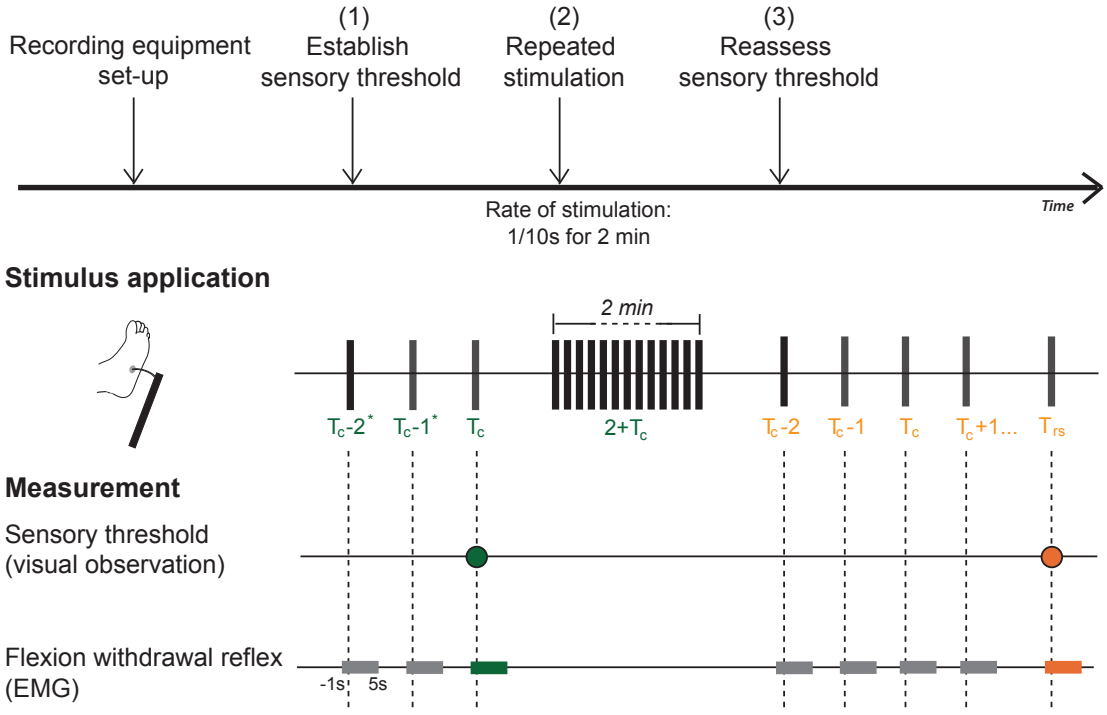


Figure 4-1: Experimental time-line

(1) Sensory threshold was established using graded stimulation of the heel; the starting forces used are shown in Table 4-2 below. (2) A series of repeated stimuli at suprathreshold force were applied once every 10s for 2 minutes. (3) Sensory threshold was reassessed after repeated stimulation; the starting forces used were 2 grades below T_c and were applied with increasing intensity until T_{rs} was observed. Key: **T_c**, control sensory threshold; **T_{rs}**, sensory threshold post-repetitive stimulation; **T-2**, force applied 2 grades below T; **T-1**, force applied 1 grade below T. Vertical black lines indicate each mechanical stimulus application; dotted lines indicate the time of stimulus for threshold assessment and EMG analysis; coloured circles represent the sensory threshold, coloured blocks represent the recording epoch used in EMG data analysis (epoch size -1s before the stimulus and 5s after the stimulus; grey = subthreshold, green = control threshold, orange = threshold post-repetitive stimulation). *post-hoc labelling, actual forces used were based on previous sensory threshold testing in human infants (Andrews *et al.*, 1999) as shown in Table 4-2 below.

GA (weeks)	Expected threshold (vFh number)[1]	Force (g)	Starting intensity (vFh number)	Force (g)
28-<33	7	0.47	5	0.13
33-<37	8	0.75	6	0.30
37-43	9	1.23	7	0.47

Table 4-2: Actual starting forces used to establish T_c

[1]Expected threshold based on previous work by Andrews and colleagues (1999)

4.3.3 Recording

The protocol for EMG recording is described in the general methods 2.6.1. Twenty studies were conducted with ipsilateral EMG recordings of biceps femoris muscle activity for analysis of flexion withdrawal reflex activity.

4.3.4 Data analysis

4.3.4.1 Sensory threshold (T)

A clear reflex response to heel stimulation was defined by:

Visual observation: The lowest grade of von Frey hair required to evoke a visible clear, brisk withdrawal of the limb at the time of the study.

EMG analysis: A change in EMG activity that exceeded 3 SD of baseline activity when analysed after the study finished.

4.3.4.2 EMG analysis

EMG analysis is described in detail in the General Methods (see section 2.7.1). Baseline activity (-1000 to time 0) was stable and there were no significant differences in mean baseline activity between stimuli or across gestational age. The reflex response was quantified by:

(1) Latency: Onset latency (ms) and Peak latency (ms)

(2) The pattern of activity: The two methods optimised in Study 1 (Chapter 3) were used to assess the pattern of EMG activity. (1) *Non-latency corrected analysis* was used to validate visual observations using EMG recordings at subthreshold and threshold stimuli; (2) *Latency-corrected analysis* adjusts for variation in the onset of the response and is a more appropriate method for determining how the pattern of EMG activity changes once the reflex response is evoked; thus comparisons of the pattern of actual reflex activity between testing groups could be made e.g. preterm versus full-term at $\mathbf{T_c}$.

(3) Mean amplitude over 2000ms: As above, the two methods optimised in Study 1 were used to summarise EMG activity over a single 2000ms time-bin where appropriate. (1) *Non-latency corrected analysis* (the root mean square of EMG activity (μV) measured in a single 2000ms time-bin from the time of stimulus to 2000ms after the stimulus) was calculated to

summarise subthreshold EMG activity. (2) *Latency-corrected analysis* (the root mean square of EMG activity (μV) measured in a single 2000ms time-bin from the time of onset of activity to 2000ms after the onset of activity) and was calculated to summarise EMG activity at threshold forces.

(4) After-stimulus discharge: To assess the effect of repeated stimulation on evoked reflex EMG activity the EMG response was separated into two parts: (1) *'evoked response (EvR)'* – where non-latency corrected analysis was used to calculate the RMS of EMG activity in a single time bin from the time of stimulus to 2000ms after the stimulus, and (2) the *'after-stimulus discharge (AD)'* – where the RMS of EMG activity between 2000ms-4000ms after the stimulus was calculated.

4.3.5 Statistical analysis

Statistical analysis is described in detail in the General Methods (see section 2.8). Statistical testing was performed as follows:

(1) Sensory threshold was compared using a Students t-test for (1) gestational age (GA) - preterm versus full-term (unpaired t-test), (2) the effect of repeated stimuli- $\mathbf{T_c}$ versus $\mathbf{T_{rs}}$ (paired t-test). (3) For testing the relationship between $\mathbf{T_c}$ and individual GA, a Pearson's correlation test was performed, following positive testing for a Gaussian distribution. (4) For testing the relationship between $\mathbf{T_{rs}}$ and individual GA, a Spearman's correlation test was performed following negative testing for a Gaussian distribution.

(2) Latency to onset of reflex response, and peak activity, were compared also using a *Student's t-test* for (1) age (unpaired t-test), and (2) the effect of repeated stimuli- $\mathbf{T_c}$ versus $\mathbf{T_{rs}}$ in preterm infants (Wilcoxen matched pairs test) and for full-term infants (paired t-test).

(3) The pattern of activity was compared for (1) force applied – subthreshold versus $\mathbf{T_c}$, using a *one-way ANOVA with repeated measures and Bonferroni post-hoc* testing. (2) gestational age at threshold- preterm versus full-term infants using a *two way-ANOVA with repeated measures and Bonferroni post-hoc testing*. (3) the effect of repeated stimuli for each age group- $\mathbf{T_c}$ versus $\mathbf{T_{rs}}$ *using a two way-ANOVA with repeated measures and Bonferroni post-hoc testing.*

(4) The mean activity over 2000ms was compared (1) force applied – subthreshold (1-T, 2-T) versus threshold, using a *one-way ANOVA with repeated measures and Bonferroni post-hoc* testing. (2) gestational age at threshold- preterm versus full-term infants using *Mann-Whitney* testing.

4.4 Results

Twenty studies were conducted in preterm and full-term infants to establish flexion withdrawal reflex activity at sensory threshold and subthreshold forces. The EMG recording was analysed after the study to validate observational methods and quantify evoked motor activity at threshold (**T_c, T_{rs}**), 1 below threshold (**T_c-1, T_{rs}-1**) and 2 below threshold (**T_c-2, T_{rs}-2**) forces.

Nineteen studies were included in the final EMG analysis; 12 preterm and 7 full-term infants. Table 4-3 refers to the infant demographics of the group. One full-term infant was excluded from analysis because the EMG recording was contaminated with 50Hz activity.

	Preterm (N=12)	Full-term (N=7)
Male; (n/N)	67%; 8/12	29%; 2/7
Mean GA at birth (weeks)	29.92±3.82; range 24.14-35.14	33.08±5.85; range 26.71-40.86
Mean GA at study (weeks)	33.50±2.29; range 30.14-36.00	38.71±1.69; range 37.29-42.29
Mean PNA (days)	25.00±18.37; range 6 -53	39.43±31.76; range 5-74
Mean weight at study (g)	1768.67±750.88; range 732.00-2700.00	2348.86±576.94; range 1920.00-3564.00
Right heel stimulated; (n/N)	67%; 8/12	43%; 3/7

Table 4-3: Infant demographics
Data given as mean± standard deviation unless otherwise stated

4.4.1 Validation of visual observation to detect sensory threshold using EMG characterisation of flexion withdrawal reflex activity in full-term infants

Sensory thresholds, as detected by visual observation of limb movement, were validated using EMG analysis in full-term infants; the pattern of EMG activity and mean activity over 2000ms

were compared at threshold and subthreshold forces. The force of von Frey that evoked a clear, observable brisk limb movement when applied to the heel was defined as threshold ($\mathbf{T_c}$).

In full-term infants (n=7), mean cutaneous sensory threshold was 1.79g (95% CI 1.03-2.55g). Analysis of the EMG recording was performed post-hoc. Example EMG recordings following stimulus application at sensory threshold ($\mathbf{T_c}$) and subthreshold force ($\mathbf{T_c\text{-}2}$, $\mathbf{T_c\text{-}1}$) are shown in Figure 4-2.

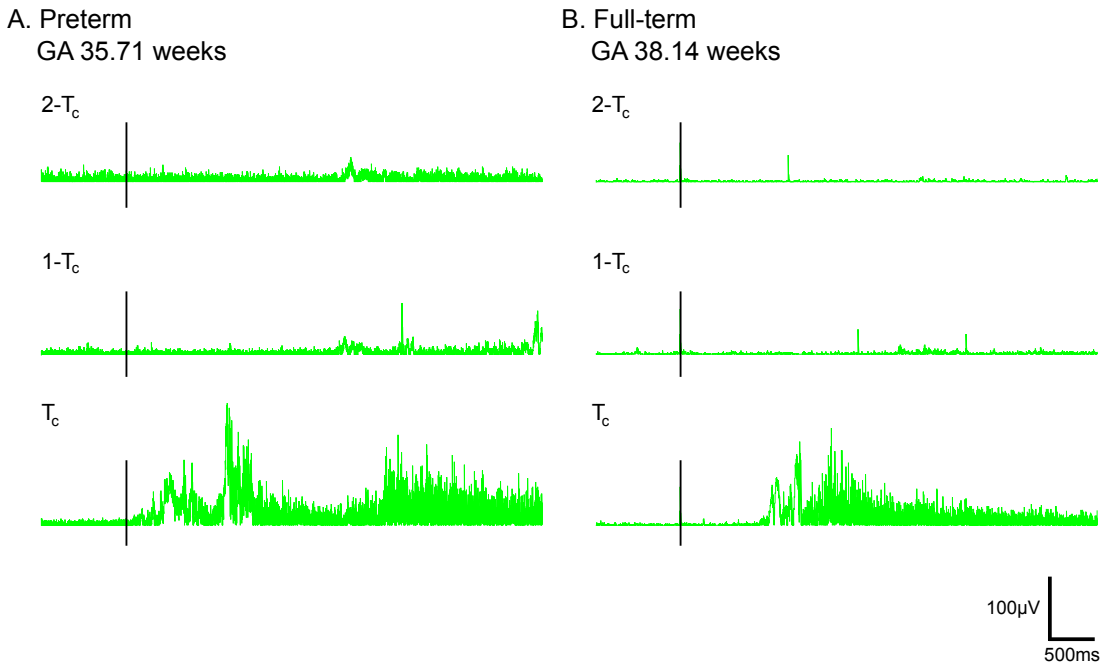


Figure 4-2: Example EMG recordings from (A) preterm and (B) full-term infant after subthreshold and threshold mechanical stimulation
EMG activity at forces 2 grades below threshold (**2-T_c**); middle traces indicate EMG activity using forces at 1 hair below threshold (**1-T_c**); bottom traces indicate sensory threshold (**T_c**) where clear brisk withdrawal of the limb was observed following stimulus. In these example recordings, both the preterm infant in (A) and full-term infant in (B) responded to vFh number 9, force of 1.23g, but not to vFh number 7 (2-T_c; force of 0.47g) or vFh number 8 (1-T_c; force of 0.75g). Vertical black line indicates time of stimulus application; scale bar is located at the bottom right of figure. Note: event-marking artefact present in full-term infant- this is characterised by a sharp single spike.

4.4.1.1 Pattern of EMG activity

The pattern of mean EMG activity was analysed using non-latency corrected analysis. The pattern of activity for individual infants at threshold (Figure 4-3 on page 145) reflects the variation in the reflex response in the sample, particularly drawing attention to one infant with gross activity at 500-750ms that potentiates the overall mean activity at this time window. The group average pattern of EMG activity at threshold and subthreshold is shown in Figure 4-4.

At threshold ($\mathbf{T_c}$), a rapid increase flexion withdrawal reflex activity occurred between 500ms and 750ms after the stimulus. Mean peak activity was 31.12µV (95% CI -5.91-68.15µV) between 500 and 750ms after the stimulus. EMG activity decreased slightly to 23.44µV (95% CI 9.82-37.06µV) between 1000ms and 1250ms and remained sustained at this level for at least 1500ms before steadily decreasing towards baseline levels. At subthreshold forces the EMG activity was not evoked by the stimulus. As Figure 4-4 shows, there was no increase in subthreshold group mean pattern of EMG activity over the 5s recording period after the stimulus.

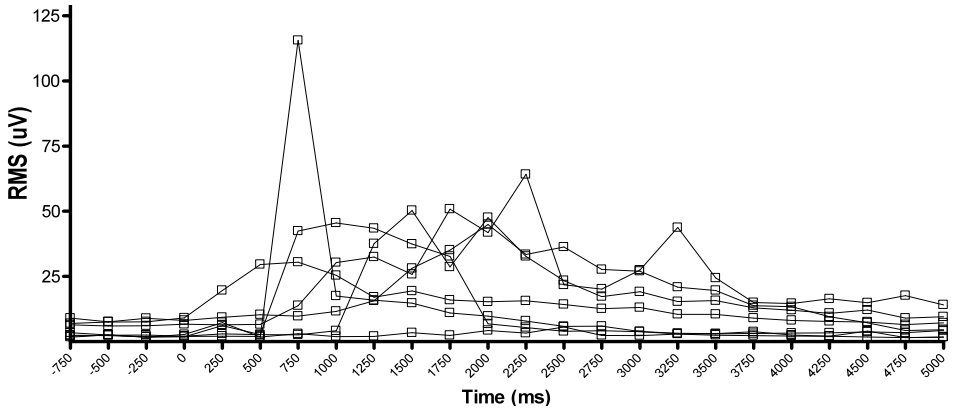


Figure 4-3: Ipsilateral biceps femoris activity of individual full-term infants at threshold force
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 5000ms. The stimulus was applied at t0

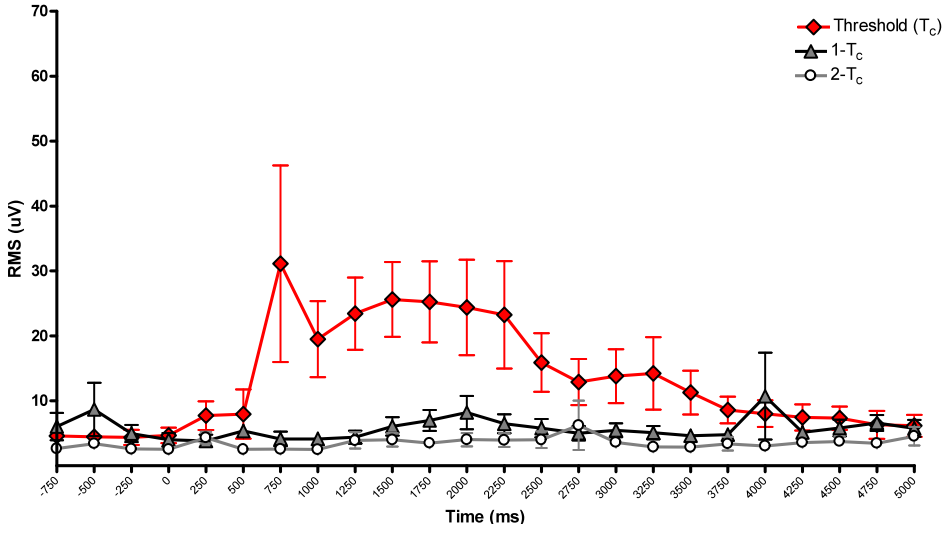


Figure 4-4: Group mean ipsilateral biceps femoris activity of full-term infants at subthreshold and threshold forces
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins. between -1000ms and 5000ms. The stimulus was applied at time 0. EMG activity evoked threshold force is shown in red.

4.4.2 Mean activity over 2000ms

Mean activity over 2000ms was determined to summarise evoked flexion withdrawal reflex activity at threshold ($\mathbf{T_c}$) and subthreshold. At threshold, mean activity over 2000ms from the onset of the response was 29.34μV (95% CI 17.24-41.43μV). This was significantly larger than mean activity over 2000ms, measured from the same time point, at subthreshold: $\mathbf{T_c}$**-1**, 7.45μV (95% CI 3.01-11.89μV), and $\mathbf{T_c}$**-2**, 3.62μV (95% CI 1.54-5.70μV). Figure 4-5 shows these differences were significant (ANOVA with repeated measures: $F_{2,8}$=13.89; p=0.001; Bonferroni post-hoc testing for $\mathbf{T_c}$ vs **2-**$\mathbf{T_c}$, p=0.00l; $\mathbf{T_c}$ vs **1-**$\mathbf{T_c}$, p=0.001). The activity evoked at **2-**$\mathbf{T_c}$ and **1-**$\mathbf{T_c}$ was not significantly different from baseline.

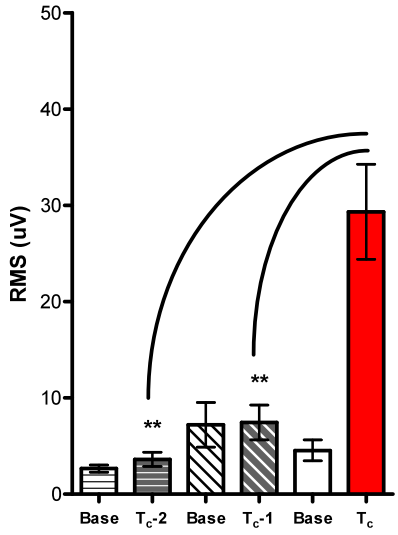


Figure 4-5: Validation of visual observation methods to detect sensory threshold (T_c) activity in full-term infants
Sensory threshold was established using visual observation of lower limb activity. EMG analysis of biceps femoris activity indicated that significant EMG activity was evoked at threshold compared to subthreshold forces ($\mathbf{T_c}$**-2** and $\mathbf{T_c}$**-1**). Base, mean baseline activity (RMS from -1000 to time 0) prior to each stimulus application. Asterisks' indicate level of significance: **, p<0.001.

4.4.3 Validation of visual observation to detect sensory threshold using EMG characterisation of flexion withdrawal reflex activity in preterm infants

In preterm infants (n=12), the mean cutaneous sensory threshold was 1.04g (95% CI 0.65-1.42g) and evoked clear, brisk withdrawal of the limb. Example EMG recordings following

stimulus application at sensory threshold ($\mathbf{T_c}$) force and subthreshold ($\mathbf{T_c\text{-}2}$, $\mathbf{T_c\text{-}1}$) and are shown in Figure 4-2 on page 144.

4.4.3.1 Pattern of activity

The pattern of EMG activity for individual infants at threshold is shown in Figure 4-6. Group mean EMG activity at threshold and subthreshold force in shown in Figure 4-7.

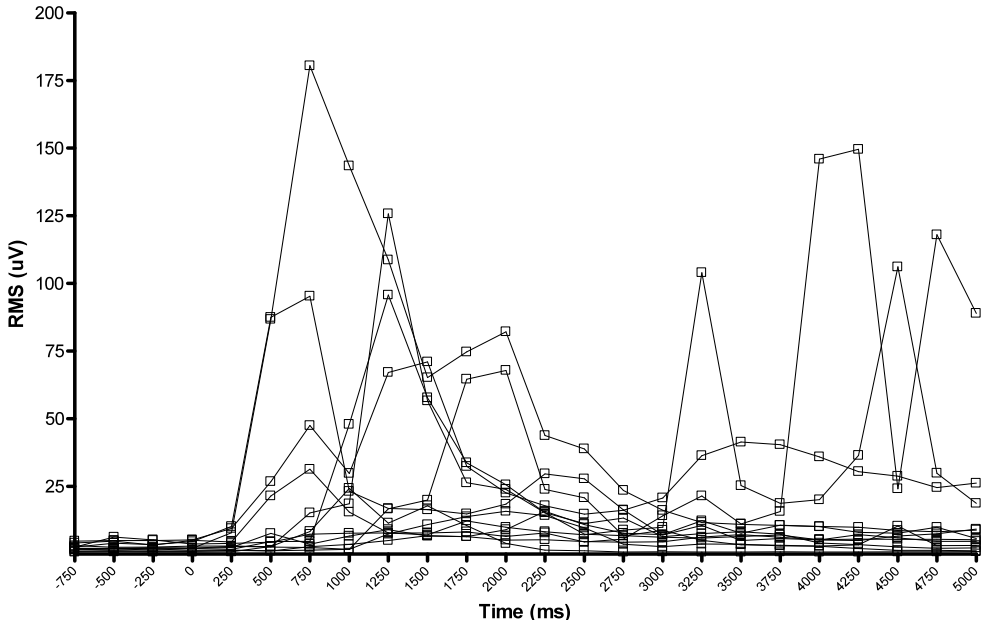


Figure 4-6: Ipsialateral biceps femoris activity of individual preterm infants at threshold force
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 5000ms. The noxious stimulus was applied at time 0.

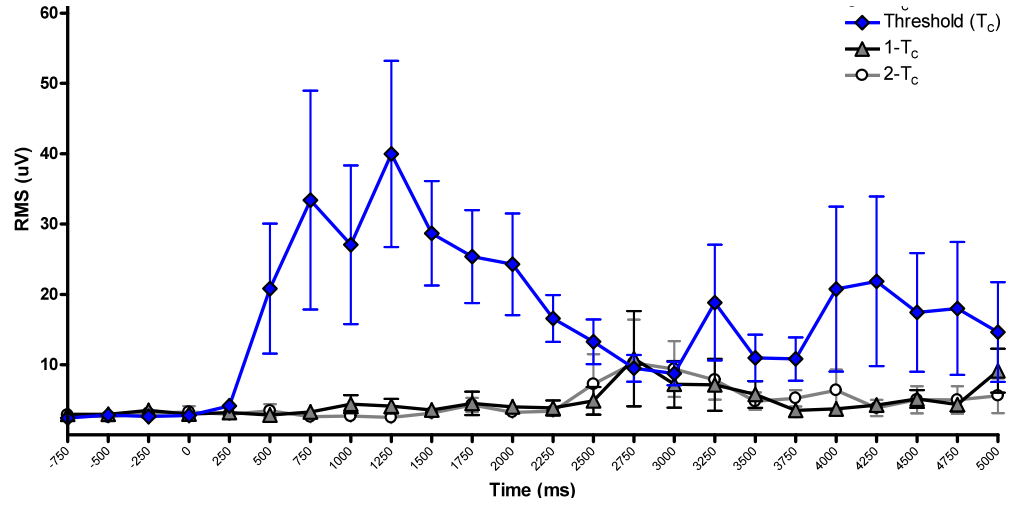


Figure 4-7: Group mean ipsilateral biceps femoris activity of preterm infants at subthreshold and threshold forces
The RMS mean (±standard error) of each time bin is shown. EMG activity at threshold force is shown in blue.

At threshold, the mean evoked EMG response was characterised by a gradual rise in activity that peaked between 1000 and 1250ms after the stimulus. Peak activity measured 39.97μV (95% CI 10.83-69.11μV). Activity steadily decreased and by 2000ms had fallen by 39% of peak activity to 24.27μV (95% CI 8.36-40.18μV). Activity remained above baseline levels for the duration of the recording period.

4.4.3.2 Mean activity over 2000ms

At threshold ($\mathbf{T_c}$), the mean activity over 2000ms from time of onset of EMG activity (latency-corrected) was 35.58μV (95% CI 17.63-53.53μV). This EMG activity was much larger than the subthreshold activity: **T_c-1**, 3.98μV (95% CI 1.93-6.03μV), and **T_c-2**, 3.27μV (95% CI 1.74-4.80μV). As Figure 4-8 shows, these differences were significant when compared (ANOVA, $F_{2,31}$=12.45; p=0.0001; Bonferroni post-hoc testing for $\mathbf{T_c}$ versus **2-T_c**, p<0.001; for $\mathbf{T_c}$ versus **1-T_c**, p<0.001). The activity evoked at **2-T_c** and **1-T_c** was not significantly different from baseline.

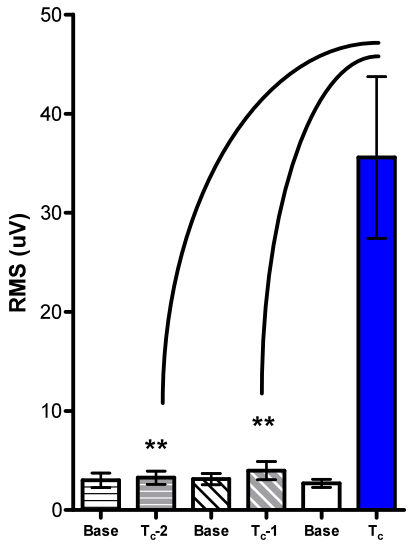


Figure 4-8: Validation of behavioural observation to detect sensory threshold (T) activity in preterm infants
Sensory threshold was established during the study using visual observation of lower limb activity. The force of the lightest vFh required to evoke a clear, brisk withdrawal of the leg was defined as the sensory threshold. EMG analysis of biceps femoris activity indicates that significant EMG activity was evoked at threshold compared to subthreshold forces (**T_c-2** and **T_c-1**). Base, mean baseline activity (RMS from -1000 to time 0) prior to each stimulus application. Astrisks indicate significance level: **, p<0.001.

Summary 1

This set of analyses has validated the use of visual observations to detect sensory threshold in preterm and full-term infants using EMG recordings of flexion withdrawal reflex activity. EMG recordings provided additional information regarding specific motor activity characteristics including the pattern of evoked activity. In the next section EMG measurements were used to compare reflex activity evoked at $\mathbf{T_c}$ between preterm and full-term infants.

4.4.4 Comparison of flexion reflex withdrawal activity in preterm and full-term infants

Properties of the flexion withdrawal reflex evoked by von Frey hair stimulation of the heel were compared between the two groups of infants: full-term (n=7) and preterm (n=12). The sensory threshold values and flexion withdrawal EMG properties in terms of latency, pattern of activity and mean activity over 2000ms characterised.

4.4.4.1 Behavioural observation of cutaneous sensory threshold

Preterm infants had significantly lower sensory thresholds of 1.04g (95% CI 0.65-1.42g) compared to full-term infants, 1.79g (95% CI 1.03-2.55g); unpaired t-test, p=0.04. The threshold force capable of evoking a clear, brisk withdrawal of the limb for each individual infant was plotted against GA. As Figure 4-9 shows, the size of mechanical force required to evoke flexion withdrawal increases with gestational age. Pearson's correlation test indicated a significant positive relationship between the two values (p= 0.005; r=0.62, 95% CI 0.22-0.83).

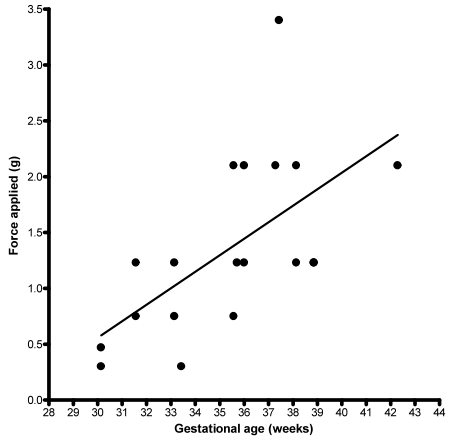


Figure 4-9: Cutaneous sensory threshold increases with gestational age
Each circle represents the lowest force applied that evoked visual observation of leg withdrawal in an individual infant. Pearson's correlation analysis indicate r=0.62.

4.4.4.2 Latency

Latency to onset of threshold flexion withdrawal reflex activity were compared at threshold ($\mathbf{T_c}$): for preterm infants, the latency was 603.3ms (95% CI 315.6-891.0ms), and for full-term infants, 871.1ms (95% CI 347.8-1394.0ms) which was not significantly different (unpaired t-test, p=0.27). Latency to peak activity was longer in preterm infants, although this was not significantly different to the time-taken in full-term infants (unpaired t-test, p=0.43); preterm, 1354.0ms (95% CI 1040.0-1668.0ms), full-term infants, 1143.0ms (95% CI 563.2-1723.0ms).

4.4.4.3 Differences in the pattern of reflex activity

Differences in the pattern of EMG reflex response were compared at threshold ($\mathbf{T_c}$) using latency-corrected analysis in preterm and full-term infants. Whilst the force required to evoke the reflex response was significantly lower in preterm infants, there were no significant differences in the corrected pattern of activity between the preterm and full-term infants ($F_{1,323}$=0.39; p=0.54); Figure 4-10. Preterm corrected peak activity occurred between 250 and 500ms after the onset of the reflex response, and activity remained sustained for a further 250ms before steadily decreasing towards baseline levels. Full-term infant corrected peak activity also occurred within the first 250ms of the reflex response but rapidly decreased in amplitude immediately afterwards.

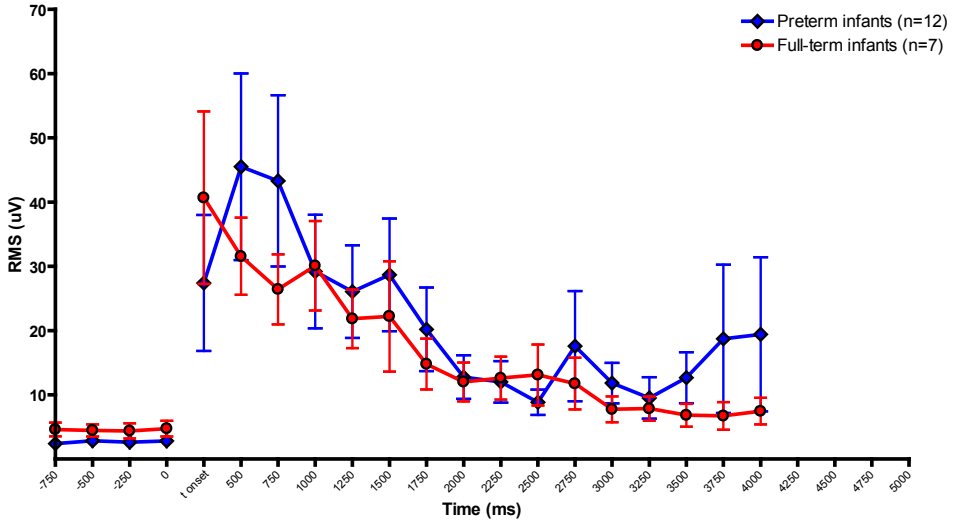


Figure 4-10: Flexion withdrawal reflex activity in preterm and term infants at threshold force
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of reflex activity (t onset) and the end of the recording epoch (in completed 250ms time bins). The RMS (±standard error) in each time bin is displayed. Activity is terminated at 3750-4000ms since the large variation in onset latency affected the number of infants included in the final time bins.

4.4.4.4 Mean activity over 2000ms

There were no significant differences in mean activity at threshold ($\mathbf{T_c}$) over 2000ms (latency-corrected) between preterm, 32.04μV (95% CI 13.38-50.69μV), compared to full-term infants, 26.38μV (95% CI 15.38-37.37μV); Mann-Whitney test; p=0.70.

Summary 2

Sensory threshold and the associated flexion withdrawal reflex EMG activity for preterm and full-term infants are summarised below in Table 4-4.

Cutaneous sensory threshold to mechanical stimulation of the heel increased with gestational age; preterm infants exhibited significantly lower sensory thresholds compared to full-term infants. Furthermore, the characteristics of evoked flexion withdrawal reflex EMG activity were compared at threshold ($\mathbf{T_c}$) and were not significantly different at any age in the parameters measured.

	Preterm infants (n=12)	Full-term infants (n=7)	p-value
Mean threshold (g) [1]	1.04 (0.65-1.42)	1.79 (1.03-2.55)	**0.04**
Mean baseline activity (μV) [2]	2.70 (1.78-3.62)	4.55 (1.90-7.21)	0.08
Latency to response (ms) [1]	603.3 (315.6-891.0)	871.1 (347.8-1394.0)	0.27
Latency to peak activity (ms) [1]	1354.0 (1040.0-1668.0)	1143.0 (563.2-1723.0)	0.43
Latency-corrected analysis			
Peak amplitude (μV) [2]	45.50 (13.53-77.46)	40.68 (7.84-73.52)	>0.05
Amplitude at 2000ms (uV) [2]	12.76 (5.29-20.23)	12.00 (4.58-19.43)	>0.05
Mean activity over 2000ms (μV) [3]	35.58 (17.63-53.53)	29.34 (17.24-41.43)	0.47

Table 4-4: Summary of flexion withdrawal reflex activity a threshold force in preterm and full-term infants
All data expressed as mean (95% CI). [1]Students unpaired t-test; [2]Two-way analysis of variance (ANOVA) with repeated measures; [3]Mann-Whitney. Significant differences are indicated in green.

4.4.5 The effect of repeated stimuli on flexion withdrawal reflex activity

The effect of repeated stimulation was investigated in twenty studies of preterm and full-term infants to examine the change in cutaneous sensitivity. Suprathreshold stimuli (two grades above control threshold; $\mathbf{T_c+2}$) were applied once every 10s for two minutes to the same site

on the foot. For ethical and clinical reasons, stimuli were not applied when body movement was evident and the infant was restless. For this reason, it was not always possible to apply stimuli every 10 seconds and, in these cases infant was allowed to stabilise before applying further stimuli to the foot. Suprathreshold forces were not noxious and did not cause visible damage to the skin or surrounding tissue. Flexion withdrawal reflex activity during the first 10 stimuli was assessed and compared between age groups.

Sixteen studies were included in the final EMG analysis; preterm (n=10) and full-term (n=6) infants. Two preterm infants were excluded because the interstimulus interval was <10s on 5 or more occassions, or the total number of stimuli applied were less than 10. Two full-term infants were excluded because the EMG recordings were contaminated with 50Hz activity or of poor quality.

4.4.5.1 Incidence

Flexion withdrawal reflex activity during the first 10 stimuli was assessed for each infant. A reflex response was defined as when evoked EMG activity exceeded 3 SD of the baseline activity. All infants responded to the suprathreshold stimulus during the train however the frequency of response was variable. Preterm infants exhibited a reflex response on 33/100 stimulus occasions (33%) and full-term infants responded on 26/60 stimulus occasions (43%).

4.4.5.2 Magnitude of reflex response during repeated stimulation

(1) Reflex activity for each stimulus applied

Only EMG recordings where a clear reflex response occurred were characterised; this was due to the variability in response incidence within both age groups. The EMG recording was divided into two 2000ms segments to determine the magnitude of the (1) evoked response (RMS over the first 2000ms after the stimulus) and (2) after-stimulus discharge (RMS between 2000ms and 4000ms after the stimulus) to see if EMG activity was maintained beyond the 2000ms post-stimulus period. Figure 4-11 illustrates the division of the EMG recording in a preterm infant. The aim here was to establish if evoked activity was sustained beyond the initial 2000ms following the stimulus during repeated stimulation.

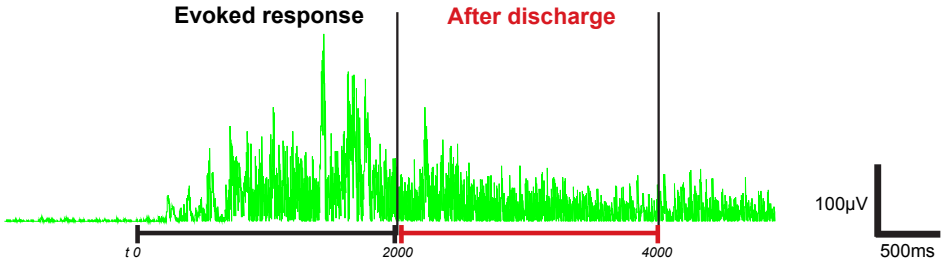


Figure 4-11: Example trace from preterm infant (33.14 weeks GA) indicating the two segments used (1) Evoked response and (2) after discharge.
The EMG recording is cut into an epoch -1000ms to 5000ms around the stimulus. The time of stimulus is indicated by 't0'. The initial period between t0 and 2000ms after the stimulus represents the 'evoked response' (black horizontal line). The later period between 2000ms and 4000ms after the stimulus is the 'after discharge' (red horizontal line). Note how in this example trace the after-stimulus discharge activity is greater than the baseline period. Scale bar is on the right hand side.

For comparison, evoked activity and after-stimulus discharge activity were also calculated at sensory threshold, $\mathbf{T_c}$, (from section 4.4.4) prior to repeated-stimulation.

In both age groups at threshold, $\mathbf{T_c}$, evoked EMG activity was consistently larger than after-stimulus discharge activity (Figure 4-12). The mean evoked response in preterm infants was 32.08µV (95% CI 9.51-54.65µV) and in full-term infants was 25.75µV (95% CI 12.22-39.28µV). The mean after-stimulus discharge activity in preterm infants was 12.27µV (95% CI 5.68-18.86µV) and in full-term infants was 13.79µV (95% CI 1.71-25.87µV). There are no significant differences these two measures between preterm and full-term infants (Kruskal-Wallis test, p=0.15).

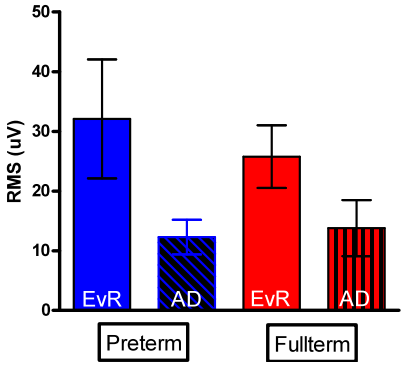


Figure 4-12: Evoked EMG response (EvR) and after-stimulus discharge (AD) activity at sensory threshold (T) in preterm and full-term infants
Preterm infants are shown in blue, full-term infants are shown in red. After-stimulus discharge activity is lower than evoked activity for both age groups. No significant differences between groups (p=0.15).

For repeated stimulation at **2+T_c**, the evoked activity and associated after-stimulus discharge were calculated for each reflex response to the stimulus. The magnitude of the evoked response is shown in Figure 4-13 for each stimulus in preterm and full-term infants. There were no clear patterns in the size of evoked response over time or between ages.

Full-term infants exhibited an increase in the size of evoked activity in the first 4 stimuli where it peaked, after this evoked activity tended to decrease in size with subsequent stimulation. After-stimulus discharge activity was determined following each stimulus in the train (Figure 4-14). Full-term infants exhibited an increase in after discharge activity with the first 4 stimuli, (accompanying the increase observed in evoked activity), after-stimulus discharge decreased in magnitude for stimuli 5-10.

Interestingly, preterm infants exhibited a build-up in the degree of after-stimulus discharge from the 4th stimulus onwards. This indicates that the level of motor activity evoked by the stimulus is maintained beyond the 2000ms after the stimulus. Of the 10 preterm infants tested, none exhibited a response to the 8th stimulus, and smaller after-stimulus discharges (<10μV) were detected at the 9th and 10th stimulus.

(2) Overall reflex activity for all stimuli

Mean evoked activity and after-stimulus discharge activity were calculated to summarise the overall activity for all responses during the period of repeated stimulation. Kruskal-Wallis testing indicated there were significant differences in the data ($p=0.002$; Duhn's post-hoc testing).

Full-term infants exhibited a significantly lower mean after-stimulus discharge activity compared to mean evoked activity ($p<0.01$). Preterm infants exhibited a heightened level of sustained activity following stimulation since there were no significant differences between mean evoked activity and mean after-stimulus discharge. No significant differences in evoked activity were found between preterm and full-term infants. The individual changes are shown in Figure 4-16.

Evoked response

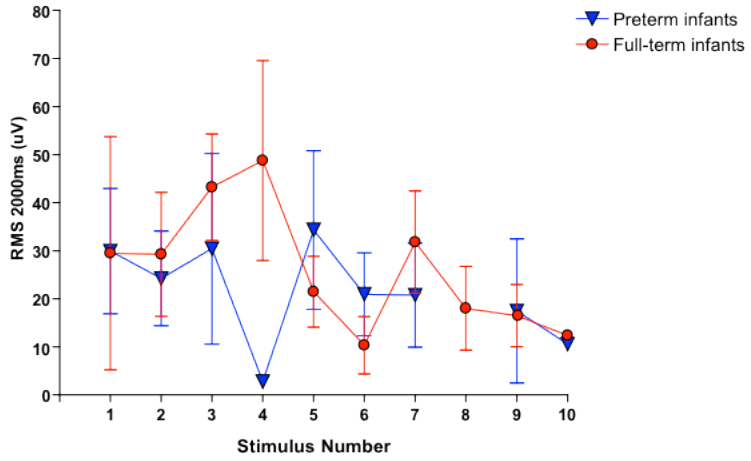


Figure 4-13: Mean evoked response for each stimulus number in preterm (n=10) and full-term (n=6) infants
Suprathreshold stimuli were applied once every 10s and the mean evoked response determined for each age group. The evoked response was measured from the stimulus (time 0) until 2000ms after the stimulus. Where no error bars are present only one infant exhibited a detectable reflex response. Note that no preterm infants exhibited a reflex response at stimulus number 8.

After-stimulus discharge

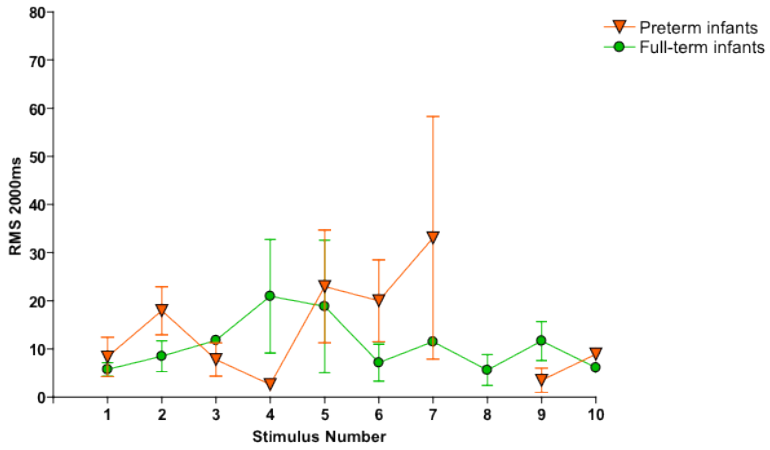


Figure 4-14: Mean after-stimulus discharge activity for each stimulus number in preterm (n=10) and full-term (n=6) infants
Suprathreshold stimuli were applied once every 10s and the mean after-stimulus discharge activity determined for each age group. The after-stimulus discharge period was between 2000ms and 4000ms after the stimulus. Where no error bars are present only one infant exhibited a detectable reflex response. Note that no preterm infants exhibited a reflex response at stimulus number 8.

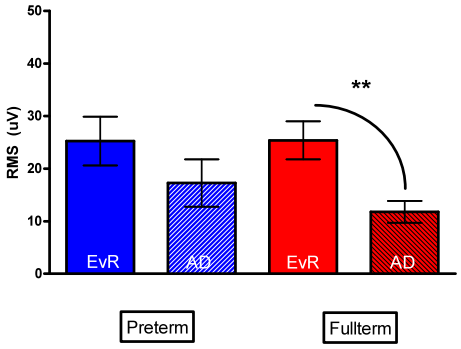


Figure 4-15: Mean evoked activity (EvR) and after-stimulus discharge (AD) for all responses evoked during repeated stimulation

Evoked activity in preterm infants (blue bar; 33/100 stimuli) and full-term infants (red bar; 26/60 stimuli) were not significantly different. The after-stimulus discharge in full-term infants (green bar) is significantly lower than evoked response in preterm and full-term infants, and the after discharge in preterm infants. These results show that preterm infants exhibit a significantly larger after-stimulus discharge after a train of repeated stimuli compared to full-term infants.

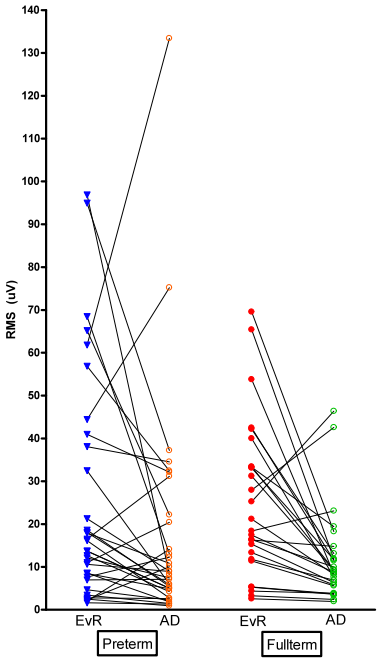


Figure 4-16: Individual evoked activity (EvR) and after-stimulus discharge (AD) for all responses evoked during repeated stimulation in preterm and full-term infants

Each point represents an individual reflex response to stimulation.

4.4.5.3 The effect of repeated stimulation on background activity

Preterm infants exhibit a sustained level of motor activity during the repeated stimuli that may be indicative of increased background activity. The aim here was to test if repeated stimulation modulated background activity after the series of repeated stimuli in preterm and full-term infants. Baseline activity was measured at two 1000ms periods before and after the series of repeated stimuli. (1) 'Pre-repetitive stimulation', the RMS between -1000ms and the time of 1st stimulus in the train; (2) 'post-repetitive stimulation', the RMS between 7000ms and 8000ms after the last stimulus in the repeated stimulation train. The time period chosen was between 7000ms and 8000ms after stimulus application as this was least likely to contain activity associated with the stimulus.

As Figure 4-17 shows, in full-term infants baseline activity was unaffected by repeated stimulation. Preterm exhibited an increase in background EMG following the repeated stimuli ('$post_{RS}$') compared to pre-repeated stimulation ('pre_{RS}'). However, no statistical significance was found between these groups (Kruskal-Wallis, p=0.69).

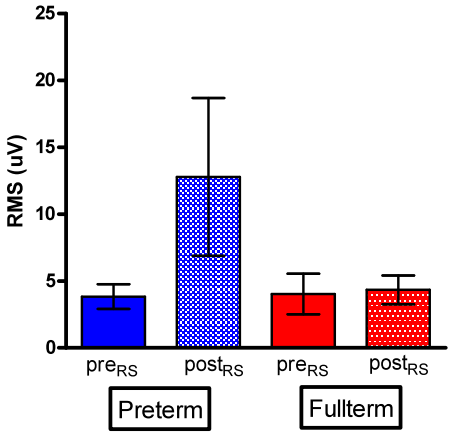


Figure 4-17: Repetitive stimulation (RS) alters background EMG activity in preterm infants
Preterm infants are shown by blue bars and full-term infants are shown in red. 'Pre-', EMG activity over a 1000ms period immediately prior to the 1st stimulus in the series of repeated stimulation, 'post', EMG activity aver a 1000ms period after the last stimulus was applied (7000ms-8000ms post-stimulus). Preterm infants exhibited larger background EMG following the repeated stimulation compared to pre-repeated stimulation (pre), however this was not significant (p=0.69).

4.4.6 The effect of repetitive stimulation at suprathreshold (T_c+2) forces on cutaneous sensitivity

The effect of repeated stimulation was investigated in twenty studies of preterm and full-term infants to examine the change in cutaneous sensitivity. Suprathreshold stimuli (two grades above control threshold; $\mathbf{T_c+2}$) were applied once every 10s for two minutes to the same site on the foot. Cutaneous threshold was re-established immediately after the last stimulus in the train using visual observations of limb movement and the associated EMG activity quantified.

4.4.6.1 The change in cutaneous sensory threshold following repetitive non-noxious stimulation (T_{rs}) in preterm and full-term infants

Cutaneous sensory threshold was established using methods described previously in this chapter. The sample was divided into full-term and preterm GA and the difference in threshold force that evoked a clear, brisk withdrawal of the limb between control values ($\mathbf{T_c}$) and following repetitive stimulation values ($\mathbf{T_{rs}}$) were compared.

All full-term infants exhibited a decrease in cutaneous sensory threshold following repeated stimulation. Habituation to additional mechanical stimulation was a significant effect at this age (paired t-test, p=0.048). As illustrated in Figure 4-18, $\mathbf{T_c}$ was 1.88g (95% CI 0.98-2.78g) and increased such that $\mathbf{T_{rs}}$ was 5.55g (95% CI 1.18-9.93g).

In preterm infants, the change in cutaneous sensitivity was variable as shown by an increase in threshold (n=9); no change in threshold (n=2), and a decrease in threshold (n=1). However, no significant differences in cutaneous sensitivity were determined after repeated stimulation (paired t-test, p=0.06). Control sensory threshold was 1.04g (95% CI 0.65-1.42g) and after repeated stimulation increased to 1.99g (95% CI 0.78-3.19g); see Figure 4-18.

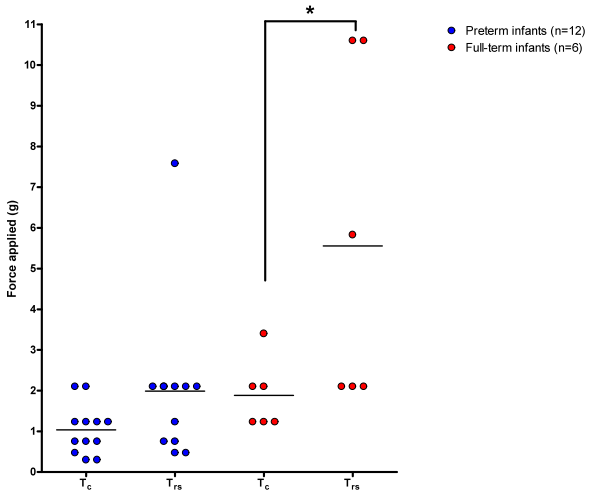


Figure 4-18: The effect of repeated stimulation on cutaneous sensitivity in preterm and full-term infants

Larger mechanical forces are required to evoke flexion withdrawal reflex activity in all infants following repetitive stimulation. Full-term infants significantly habituated to further stimulation as indicated by the astrisks; *, $p<0.05$. Each circle represents the sensory threshold of an individual infant. $\mathbf{T_c}$,control sensory threshold; $\mathbf{T_{rs}}$, post-repetitive stimulation sensory threshold.

After repeated stimulation, the threshold force ($\mathbf{T_{rs}}$) for each individual infant was plotted against GA (Figure 4-19; page 159). Habituation to additional mechanical stimulation increased with gestational age and is not significant at younger ages. The size of mechanical stimulation required to evoke withdrawal also increases with gestational age and Spearman's correlation testing indicated a significant positive relationship between the two values ($p=0.004$; $r=0.64$; 95% CI 0.22-0.86).

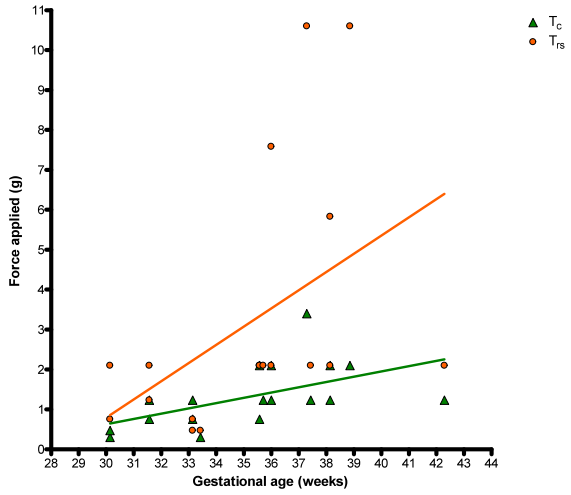


Figure 4-19: Habituation occurs following repetitive stimulation (1/10sec for 2 min) at older ages

An increase in the force required to evoke flexion withdrawal reflex activity occurred in older infants following repetitive stimulation at suprathreshold forces. Each point represents the lowest force applied that evoked behavioural observation of clear brisk leg withdrawal in an individual infant. Green line represents line of best fit for control threshold ($\mathbf{T_c}$); orange line of best fit for post-repetitive stimulation threshold ($\mathbf{T_{rs}}$).

4.4.6.2 Latency at T_{rs} compared to T_c in full-term and preterm infants

Repetitive stimulation did not affect the onset latency of the flexion withdrawal reflex in full-term or preterm infants. For full-term infants, onset latency at $\mathbf{T_{rs}}$ was 880.3ms (95% CI 191.4-1569.0ms) and was not different from $\mathbf{T_c}$, 903.8ms (95% CI 261.0-1547.0ms); paired t-test p=0.21. For preterm infants, the onset latency at $\mathbf{T_{rs}}$ was 630.9ms (95% CI 249.2-1013.0ms) and at $\mathbf{T_c}$ was 603.3ms (95% CI 315.6-891.0ms); Wilcoxen matched pairs test, p=0.85.

In addition, peak latency properties were unaffected by repetitive stimulation. For full-term infants, peak latency at $\mathbf{T_{rs}}$ was 1167.0ms (95% CI 678.2-1655.0ms) and was not different from $\mathbf{T_c}$, 1042.0ms (95% CI 390.2-1693.0ms ms); paired t-test p=0.32. For preterm infants, the onset latency at $\mathbf{T_{rs}}$ was 1167.0ms (95% CI 892.9-1440.0ms) and at $\mathbf{T_c}$ was 1354.0ms (95% CI 1040.0-1668.0ms); Wilcoxen matched pairs test, p=0.44.

4.4.6.3 The pattern of EMG activity at T_{rs} compared to T_c

The pattern of evoked flexion withdrawal reflex EMG activity was analysed at $\mathbf{T_{rs}}$ for each age group, and then compared to the response evoked at $\mathbf{T_c}$ using paired EMG recordings and latency-corrected analysis: full-term (n=6) and preterm (n=12).

In full-term infants at $\mathbf{T_{rs}}$, the evoked response was maximal over the first 750ms after the stimulus. Corrected peak activity was 43.93μV (95% CI -8.00-95.85μV), and rapidly decreased towards baseline, and did not fall below baseline over the following 3750ms (Figure 4-20). In preterm infants at $\mathbf{T_{rs}}$, the peak of the response occurred in the first 750ms after the stimulus, and measured 27.51μV (95% CI 13.96-41.07). Following this, activity decreased until 1750ms post-onset where a second wave of EMG activity occurred (Figure 4-21).

Comparison between evoked flexion withdrawal reflex activity at $\mathbf{T_c}$ and $\mathbf{T_{rs}}$ were made for each age group using corrected latency analysis. Repeated stimulation does not affect the pattern of flexion withdrawal reflex EMG activity. In full-term infants, there were no significant differences in the pattern of activity ($F_{1,210}$=0.31, p=0.59); Figure 4-20. Further, in preterm infants, there were also no significant differences ($F_{1,462}$=1.19, p=0.29); Figure 4-21.

Full-term infants

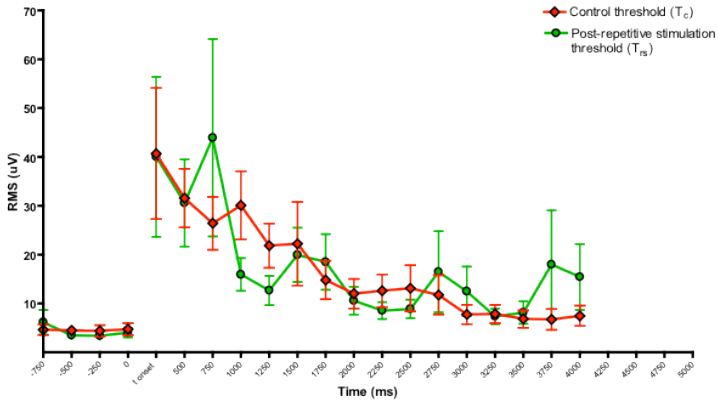


Figure 4-20: The pattern of flexion withdrawal reflex activity (corrected for latency) does not change significantly in full-term infants at threshold following repetitive stimulation
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recording epoch (in completed 250ms time bins). The RMS (±standard error) in each time bin is displayed.

Preterm infants

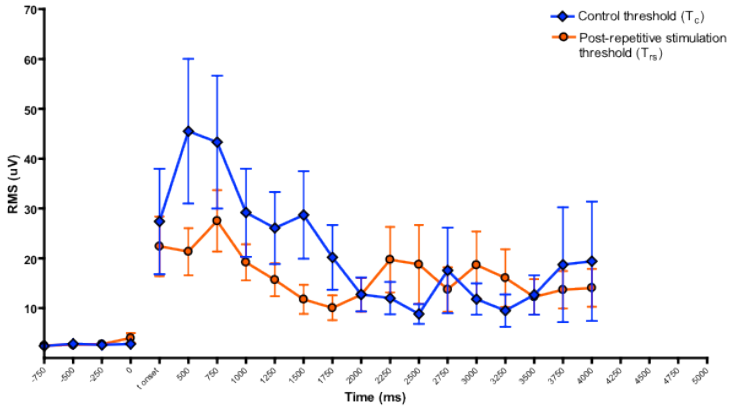


Figure 4-21: The pattern of flexion withdrawal reflex activity is not significantly attenuated in preterm infants at threshold following repetitive stimulation
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recording epoch (in completed 250ms time bins). The RMS (±standard error) in each time bin is displayed.

4.4.6.4 Mean activity over 2000ms at T_{rs} compared to T_c

Mean activity over 2000ms from onset of reflex activity (also known as the Evoked Response; 'ER') was calculated for the same paired EMG recordings used in the analysis above and again was not significantly altered by repeated stimulation.

The after-stimulus discharge activity was subsequently calculated at threshold force and compared pre- and post- repeated stimulation. As Figure 4-22 illustrates, preterm infants exhibited larger after-stimulus discharge (AD) activity following the series of repeated stimuli but this was not significantly different (One-way ANOVA, $F_{3,28}$= 0.44; p=0.73); [Preterm mean AD at $\mathbf{T_c}$ was 12.27µV (95% CI 5.68-18.89µV) and at $\mathbf{T_{rs}}$ increased to 18.46µV (95% CI 5.89-31.03µV); full-term mean AD at $\mathbf{T_c}$ was 13.79µV (95% CI 1.71-25.87µV) and at $\mathbf{T_{rs}}$ was 13.46µV (95% CI 4.53-22.40µV)

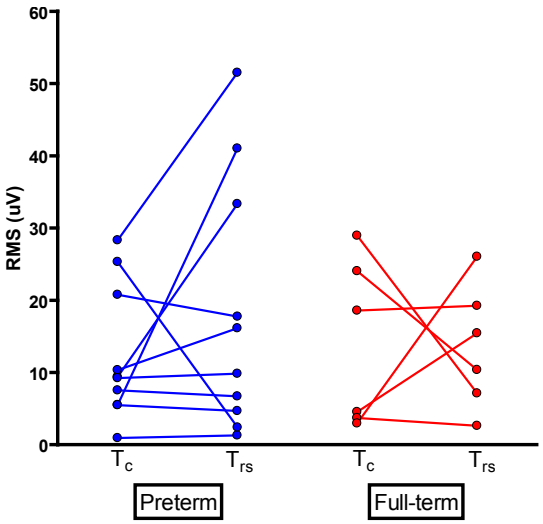


Figure 4-22: Change in after-stimulus discharge activity at threshold after repeated stimulation in preterm and full-term infants
Sensory threshold was established pre- and post repeated stimulation. After-stimulus discharge activity was calculated between 2000ms and 4000ms after the stimulus was applied. Each point represents an individual infant. Key, $\mathbf{T_c}$, control sensory threshold; $\mathbf{T_{rs}}$, post-repetitive stimulation sensory threshold.

Summary 3

Repetitive stimulation at a rate of 1/10s for 2 minutes at suprathreshold forces modulates the excitability of the flexion withdrawal reflex. The results show that preterm infants exhibit a sustained level of reflex activity during repeated stimulation, as shown by the mean after-stimulus discharge in the 2000-4000ms period after the stimulus which is not statistically different from the evoked activity. Sensory threshold testing after the series of repeated stimuli leads to habituation of activity to subsequent mechanical stimulation but this only becomes significant at older ages.

4.4.7 Summary of results

(1) Cutaneous sensory thresholds and the associated flexion withdrawal reflex EMG activity were recorded in preterm and full-term infants following single and repeated mechanical stimulation of the heel.

(2) Cutaneous sensory thresholds increase with gestational age. Preterm infants exhibit significantly higher sensitivity to mechanical stimulation compared to full-term infants.

(3) Behavioural observations of lower limb withdrawal were positively correlated with the detection of reflex activity using EMG measurement. Magnitude of EMG activity was significantly larger at threshold force compared to subthreshold forces.

(4) Repeated stimulation at suprathreshold forces (1/10s for two minutes) evoked reflex activity over the duration of the stimulus application period. Preterm infants exhibited an overall increase in after-charge activity that was not observed in full-term infants. After the train of stimuli, preterm infants showed a trend towards heightened background activity that was not observed in full-term infants.

(5) Repeated stimulation at suprathreshold forces (1/10s for two minutes) induced an age-dependent change in cutaneous sensitivity. Cutaneous sensitivity remained the same after repeated stimulation in preterm infants. However, a larger force was required to evoke the flexion withdrawal reflex after repeated stimulation compared to control threshold values in full-term infants. No differences in the flexion withdrawal reflex EMG characteristics in terms of latency and magnitude, were detected between the evoked response at control threshold and post-repeated simulation threshold.

4.5 Discussion

In this chapter EMG analysis of flexion withdrawal reflex was used to investigate the development of spinal somatosensory circuitry and the effect of repeated cutaneous stimulation in the human infant. Sensory threshold testing has been examined previously in the human infant but has depended upon behavioural observations of limb withdrawal that are subjective in nature (Abdulkader *et al.*, 2008a; Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). Firstly, the results establish that cutaneous sensory thresholds to mechanical stimulation rise with increasing GA, using quantitative EMG recordings to validate behavioural observations of limb movement. Secondly, the results show that repeated stimulation induced a change in sensitivity to subsequent mechanical stimuli that is age-dependent.

4.5.1 Effect of gestational age on initial flexion withdrawal reflex threshold

The results demonstrate that cutaneous sensory thresholds to mechanical stimulation are low in the human infant and gradually increase with gestational age; preterm infants exhibited lower sensory thresholds compared to full-term infants. These data are consistent with neonatal studies investigating cutaneous sensitivity in the kitten (Ekholm, 1967) and rat (Fitzgerald *et al.*, 1984; Fitzgerald *et al.*, 1988b; Holmberg *et al.*, 1996) using behavioural measures and direct EMG recordings of motor output. Human infant studies also support these findings (Abdulkader *et al.*, 2008a; Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). The importance of the current results are that cutaneous sensitivity measured by behavioural observations of leg movement (as used in previous reports) have been strengthened with direct, quantitative EMG recordings of motor output.

4.5.1.1 Peripheral components

Adult flexion reflex thresholds are much higher than in the neonate, and developmental studies have shown that flexion reflex thresholds increase over the postnatal period to become more adult-like in the first 2-3 weeks in the kitten (Ekholm, 1967) and rat pup (Holmberg *et al.*, 1996), and are more consistent after 37 weeks GA in the human infant (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). Furthermore, the threshold is dependent on the gestational age of the human infant rather than post-natal age as shown by sensory thresholds of preterm infants studied at full-term that were equivalent to normal full-term

infants suggesting that low sensory thresholds are characteristic of the developing reflex (Fitzgerald *et al.*, 1988b).

Lower thresholds at younger ages may be due to decreased skin thickness since a lower mechanical force would be required to activate mechanoreceptors located beneath the surface. The development of skin is hastened by birth, and an extremely premature infant can have histologically similar skin at 2-3 weeks postnatal age to that of a full-term infant (Rutter, 1988). The epidermis in full-term infants only undergoes minor changes with post-natal age and is structurally and functionally similar to the adult. Therefore skin thickness seems an unlikely reason for lower thresholds in the neonate.

Another possibility is delayed functional maturation of cutaneous sensory receptors. Primary afferent fibre activity from *in vivo* electrophysiological recordings of DRG neuron firing in the foetal rat show evoked responses to light touch, pressure, heat, and chemical irritation are detectable from E17 - E20 (Fitzgerald, 1987c). Despite the low frequency of neuronal firing compared to the adult rat, activity increased with gestational age indicating that the somatic properties were not completely functionally mature at this stage. Fitzgerald (1987a) additionally examined primary afferent properties using *in vivo* DRG recordings immediately after birth in the rat pup (P0-P14); all cutaneous mechanoreceptors were well-developed by birth and had the same response patterns and thresholds as the adult rat. Interesting, nociceptors were functional from birth – high-threshold mechanoreceptors were present from birth and responded with increasing frequencies to greater mechanical forces, and polymodal receptors responded to both intense mechanical stimulation, heat and chemical irritation of the skin. With functional stimulus/response characteristics of cutaneous receptors present from birth, the peripheral components of the nervous system cannot be important in the changes in cutaneous sensitivity observed in the neonate and centrally mediated circuitry must be involved.

4.5.1.2 Developmental maturation of dorsal horn components and descending brainstem inputs

Dorsal horn structural organisation between the neonate and adult rat are considerably disparate. Anatomical studies show the central terminals of large diameter Aß afferents that are normally located in the deeper dorsal horn can be found in the substantial gelatinosa in the neonate, and remain for up to 3 postnatal weeks in the rat (Fitzgerald *et al.*, 1994; Granmo *et al.*, 2008). Electron microscopy studies have shown that A-fibres form synaptic contacts with

cells in the substantia gelatinosa before withdrawing to laminae III-V for final termination (Coggeshall *et al.*, 1996). In the adult, Aß fibres are non-nociceptive but it is possible that in the neonate Aß mediated cutaneous afferents converge onto nociceptive circuitry and are able to evoke flexion withdrawal reflex activity earlier in development, and that this gradually becomes more suppressed as shown by lower reflex thresholds. C-fos, a member of the immediate early gene family (see Coggeshall, 2005 for comprehensive review), has been used as a tool to study nociceptive-specific activity in adult spinal pathways, since Hunt and colleagues (1987) originally found c-fos was selectively upregulated in the dorsal horn following noxious stimulation and later work by Ma *et al* (1996) showed that c-fos expression is predominantly evoked by high threshold A∂ and C afferent stimulation. Jennings *et al* (1998) showed that in the neonatal spinal cord, low threshold A-fibres are able to activate pathways in the superficial dorsal horn that in the adult are predominantly nociceptive; a noxious pinch produced c-fos expression that was independent of postnatal age (P0 vs P21) whilst low intensity touch produced an age-dependent c-fos response – that at P3 was 60% of the pinch response, at P10 was 27% and at P21 completely disappeared. Further, direct recordings of dorsal horn cell activity show that low-threshold A-fibre evoked responses to the superficial and deep dorsal horns is robustly evoked from P3 – P21 (Jennings *et al.*, 1998). The human neonate electrical flexion withdrawal reflex thresholds is closer to the threshold for the tactile flexion reflex (RII) in the adult, which suggests the involvement of Aß fibres (Andrews *et al.*, 1999). Organisation of synaptic connections in the dorsal horn undergo significant reorganisation over the postnatal period since the immature nervous system is less selective in distinguishing low-intensity stimuli from noxious input. It is possible that superficially located Aß fibres activate dorsal horn neurons that are normally involved in the nociceptive reflex pathway.

Despite functional cutaneous receptors and dorsal horn activity from birth, the rat spinal cord is still immature. *In vivo* electrophysiological recordings in the rat pup show that dorsal horn cell activity is immature from birth with low frequency responses to cutaneous stimulation, longer duration action potentials and prolonged after-stimulus discharge (Fitzgerald, 1985). Furthermore, receptive fields of dorsal horn cells are larger at birth and decrease with postnatal age (Bremner *et al.*, 2008; Fitzgerald, 1985; Jennings *et al.*, 1998; Torsney *et al.*, 2002). Substantial changes in dorsal horn inhibition occur over the postnatal period (Baccei *et al.*, 2004; Keller *et al.*, 2001). An altered balance between excitation and inhibition occurs in the neonatal period and the maturation of descending inputs is contributory (Fitzgerald *et al.*, 1986; Hathway *et al.*, 2006a). Of particular interest, a developmental change from excitation

to inhibition of spinal flexion withdrawal reflex activity after the 3rd postnatal week in the rat has been shown using direct stimulation of the RVM (Hathway *et al.*, 2009). These results suggest that brainstem circuitry is immature before the 3rd post-natal week in the rat. Supraspinal centers are important in maintaining the integrity and proper excitability of adult spinal reflex circuits, but also for functionally tuning these circuits during postnatal development (Levinsson *et al.*, 1999a). Immature synaptic coupling between primary afferent terminals and central circuitry, and a lack of inhibition in the dorsal horn from inhibitory interneurons (Fitzgerald, 1985) and descending modulation (Fitzgerald *et al.*, 1986) have been proposed to potentiate dorsal horn cell excitability. A lack of inhibition from descending inputs onto the spinal cord may contribute to the increased spinal excitability observed in the current results (Hathway et al., 2009).

4.5.1.3 Ventral horn motor neurons

Motorneurons represent the last stage of the reflex arc and control the contraction of skeletal muscles. Motorneurons begin to act functional long before DRG sensory axons and descending inputs reach the spinal cord. Efferent output underlying the motor components of the stretch reflex, which uses the same set of ventral horn motor neurons as those in hind-limb withdrawal, are functional in the rat from P0 (Kudo *et al.*, 1985). However, neonatal motor neurons are more excitable to synaptic input than the adult. Neonatal motorneurons are small in size and exhibit a high input resistance (Vinay *et al.*, 2000) and so a smaller current is required to initiate cell firing. *In vitro* electrophysiological studies show neonatal motorneurons exhibit a slower rate of firing frequency (Fulton *et al.*, 1986; Walton *et al.*, 1986). This is accompanied by a less accurate representation of sensorimotor execution in neonates compared to adults. Waldenström showed from P1 to P7 noxious thermal stimulation often produced inappropriate responses including movement towards the stimulus, and by P20 to P25 however, the same stimulus reliable evoked an appropriate adult-like movement away from the stimulus. Further, motor output has been shown to be exaggerated and uncoordinated following noxious stimuli in the kitten (Ekholm, 1967), rat (Holmberg *et al.*, 1996) and human infant (Andrews *et al.*, 1994; Andrews *et al.*, 2002a; Andrews *et al.*, 2002b). The development of motor coordination following noxious and innocuous stimulation was discussed in the previous chapter (see Chapter 3; Study 1).

4.5.2 Stimulus-response characteristics of the flexion withdrawal reflex

Cutaneous sensory threshold was detected where limb flexion movement was observed. The relationship between visual observation of behaviour and detection of reflex activity using EMG measurement were validated by comparing EMG activity at subthreshold and threshold forces. EMG properties including the pattern of activity and mean activity over 2000ms provided additional information for comparison between age groups and the effect of repetitive stimulation. Thus indicating the usefulness of EMG recordings to provide quantitative measurement of sensory processing in the neonate.

These results show that larger mechanical forces applied to the skin evoked a significantly greater flexion withdrawal reflex EMG response. This is consistent with other studies in the human adult (Chan *et al.*, 1985; Hagbarth, 1952) and neonate (Andrews *et al.*, 1999), and demonstrates that despite the variability seen in the reflex response characteristics, EMG activity is a good measure of afferent input. The current study assessed flexion withdrawal reflex responses to sub-threshold and threshold stimuli; however quantitative measurement of stimulus/response characteristics to graded stimuli i.e. 2x threshold versus noxious stimuli, and to different stimulus modalities require further study in the neonate.

4.5.3 Effect of repeated stimulation

The effect of repeated stimulation was examined in preterm and full-term infants. The present results show that preterm infants exhibit a sustained level of reflex activity during repeated stimulation, as shown by the mean after-stimulus discharge in the 2000-4000ms period after the stimulus. Repeated stimulation at suprathreshold forces (intensity: $\mathbf{2+T_c}$; frequency: 1/10s for two minutes) induced an altered sensitivity to subsequent mechanical stimuli that was age-dependent. Full-term infants ($\geq$ 37 weeks GA) habituated to additional stimulation where a significantly larger force was required to evoke the flexion withdrawal reflex after repeated stimulation compared to control threshold values. Meanwhile, preterm infants (<37 weeks GA) were unaffected by repetitive stimuli and exhibited more variable changes in subsequent sensory threshold; this was shown by an increase, decrease or maintenance of the same level of cutaneous sensitivity.

The present results are in agreement with previous studies investigating the effect of repeated stimulation in the human infant (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b). Fitzgerald and colleagues (1988b) were the first group to show age-dependent

changes in reflex withdrawal incidence during repeated stimulation using the number of visible leg withdrawals as an indicator of sensitivity; in the youngest infants (<29 weeks GA) a build-up of the flexion withdrawal reflex response occurred as spinal excitability increased, where as older infants habituated to further stimuli. Andrews (1994) demonstrated that preterm infants exhibited lower cutaneous sensory thresholds whereas full-term infants habituated to subsequent stimuli following repeated stimulation. The present results in this chapter are supported by the findings of Andrews and Fitzgerald and additionally provide quantitative analysis of flexion withdrawal reflex EMG activity for preterm and full-term infants.

These results are likely to reflect the behaviour of immature dorsal horn cells. In-vivo electrophysiological recordings of rat dorsal horn cell activity show that repeated stimulation of cutaneous A fibres at twice the threshold level does not significantly alter the magnitude of the evoked response but leds to a prolonged after-discharge beyond the stimulation period (Jennings *et al.*, 1998). These researchers showed that at postnatal day 6, 33 % of dorsal horn cells were sensitized, displaying a mean after-discharge lasting 71s, at P10, only 6 % were sensitized, with a mean after-discharge lasting 63s, and by P21, sensitization was no longer observed (Jennings & Fitzgerald, 1998). Thus postsynaptic activity evoked in neonatal rat dorsal horn cells by cutaneous afferents differs considerably from that in adults. This may be due to immaturity of interneuronal connections in dorsal horn and of descending inhibitory connections (Bremner *et al.*, 2008; Fitzgerald, 2005; Hathway *et al.*, 2009).

The increase in sensory threshold seen in older, full-term infants is a characteristic change also observed in adult humans. Habituation is defined as the decrease in synaptic response that occurs as a stimulus train proceeds; it can occur over minutes of repeated stimulation at low frequency with recovery occurring over minutes to months (see Mendell, 1984 for review). Dimitrijevic and Nathan (1970) were the first to demonstrate habituation in the adult man using a series of electrical stimuli. Bromm and Scharein (1982) later reported that repeated electrical evoked finger withdrawals decreased in EMG amplitude with increasing stimulus train. Habituation of the flexion withdrawal reflex can be evoked in the human adult with repetitive electrical stimulation as shown by a gradual decrease in reflex activity (Dimitrijevic *et al.*, 1972; Dimitrijević *et al.*, 1970; Shahani *et al.*, 1971). Animal and human studies confirm habituation can be evoked by electrical stimulation and thus the mechanisms underlying habituation are unlikely to involve cutaneous receptor adaptation and are centrally-mediated (Christoffersen, 1997). Indeed, electrophysiology studies in the adult spinalised cat

have shown a reduction in ventral horn motorneurons firing occurs with repeated electrical stimulation to the hind paw and the authors' postulate the changes may be a result of prolonged hyperpolarisation in primary afferent terminal endings (Buchwald *et al.*, 1965). Theories of habituation postulate that (1) gradual reduction in the amount of excitatory neurotransmitter release by each stimulus or desensitisation of the post-synaptic membrane leads to reduced synaptic activity and/or (2) a build-up of inhibition are responsible. The former proposes that decreased neurotransmitter release, or receptor saturation and subsequent decreased sensitivity to the neurotransmitter, are contributory. The latter hypothesis postulates an increase in either presynaptic or postsynaptic inhibition in the spinal reflex arc arises. Spencer *et al* (1966) measured intracellular post-synaptic potentials in motor neurons of spinalised cats and observed synaptic depression further confirming the involvement of centrally-mediated mechanisms. In support of these studies, Wickelgren (1967a; 1967b), confirmed the lack of involvement of changes in motor neurons and observed depression of spinal interneuronal activity. It is possible that repeated afferent stimulation leads to a build-up in inhibition of spinal interneurons projecting to motor neurons, in turn causing the motor neurons to discharge fewer and fewer impulses as the repeated stimulation proceeded. The findings that habituation can be diminished by strychnine, which blocks inhibition by antagonism of glycine receptors, supports this hypothesis (Pearson *et al.*, 1973). Moreover, blocking descending input from higher centres prevented habituation of activity in lamina V cells (Wall, 1967). It is possible that habituation of the flexor reflex in full-term infants is partly due to inhibition of interneuronal connections in the deep dorsal horn and the influence of supraspinal centres.

Preterm infants exhibited no significant changes in sensory threshold in the present study. Previous work has showed that a drop of 10-20% in preterm infants follows repeated stimulation (Andrews *et al.*, 1994). Likewise, repeated stimulation in the neonatal rat leads to a drop of 60% over the first 3 postnatal weeks (Fitzgerald *et al.*, 1988b). These results, in combination with a decrease in the number of withdrawal reflex responses observed (Andrews *et al.*, 1999; Fitzgerald *et al.*, 1988b); clearly show that the developing nervous system is more susceptible to changes in sensitivity at a younger age. Although the current study design was based on the methodology used by these investigators with regard to stimulus intensity and frequency of repeated application, but a significant decrease in sensory threshold was not observed in preterm infants. However, the present results do suggest that repeated stimulation evoke an increase in after-stimulus discharge activity that is specific to preterm infants. The difference in these human data compared to the present results is possibly due to

small numbers of infants studied at very young ages. Furthermore, since hyperalgesia is a factor known to lower sensory thresholds in the rat (Woolf, 1983) and human infant (Fitzgerald *et al.*, 1988a; Fitzgerald *et al.*, 1989), it is possible that differences in the number and recent exposure to invasive procedures such as heel lancing may be an influencing factor between these studies.

4.5.4 Clinical implications

The current results show that human infants are hyper-responsive to low threshold, innocuous stimuli during early-life. Further, repeated stimulation over a short period of time does not lead to habituation of spinal cord circuitry as it does in older subjects and in very young infants can lead to maintained afterdischarge. Clinical studies suggest that non-painful sensory stimulation of infants, especially preterm and newborn, can produce equal or higher levels of physiological stress activation than painful stimulation (Hellerud *et al.*, 2002). Follow up studies also suggest that repetitive procedures alters the sensitivity threshold of preterm infants compared with full-term infants for at least the first year of life (Abdulkader *et al.*, 2008b) and can have long-term effects on pain perception (Hermann *et al.*, 2006). These data provide objective justification for keeping the number of stressful procedures to a minimum and highlight the need for careful planning of neonatal care at every stage in hospital care.

4.5.5 Conclusion

The results of this chapter have revealed that cutaneous sensory thresholds rise with increasing gestational age, and that repeated stimulation only leads to habituation in infants aged 37 weeks GA or older. Repeated stimulation in younger preterm infants can lead to maintained activity and increased background activity in preterm infants. This is consistent with earlier, observational human infant studies showing developmental changes in cutaneous sensitivity to single stimuli (Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b) and repeated stimuli (Andrews *et al.*, 1999; Fitzgerald *et al.*, 1988b), and may be due to the dominance of Aβ fibre excitatory inputs to the immature dorsal horn and the maturation of inhibitory synaptic transmission and descending projections from the brainstem.

Chapter 5

Study 3

Oral sucrose as a modulator
of nociceptive spinal flexion withdrawal reflex activity

5 Study 3

5.1 Introduction

Preterm and full-term infants admitted to intensive care are frequently exposed to painful and stressful procedures as part of their essential medical care (Carbajal *et al.*, 2008; Simons *et al.*, 2003). Commonly performed painful procedures are tissue damaging and include heel lancing and venipuncture, which are required for blood sampling (Carbajal *et al.*, 2008). With an average intensive care stay of 56 days the adverse consequences of untreated pain is of concern (Green *et al.*, 2005).

Research-based Consensus Statements and Guidelines are responsible for recommending appropriate evidence-based strategies for pain management in newborn infants. The International Evidence-Based Group for Neonatal Pain published recommendations of the use of oral sucrose administration for procedural pain in neonates (Anand, 2001); these guidelines were based on many randomised trials that have shown sucrose is effective in relieving pain. Despite this, controversies about the analgesic efficacy of sucrose prevail with concerns that it may not modulate central pain pathways sufficiently to relieve pain (Fitzgerald, 2009; Holsti *et al.*, 2010). Considering infants undergo numerous painful procedures during their hospital stay, and that the effects of pain in early life may have long-lasting effects on pain behaviour and infant neurodevelopment (Abdulkader *et al.*, 2008b; Hermann *et al.*, 2006; Hohmeister *et al.*, 2010), it is essential to assess the clinical effectiveness of sucrose on nociceptive processing in this vulnerable population.

5.1.1 Evidence for sucrose use

Sucrose has been shown to promote calming effects and to reduce distress associated with noxious stimuli in both animal models and humans (Bhattacharjee *et al.*, 2005; Blass *et al.*, 1987; Segato *et al.*, 1997; Stevens *et al.*, 2010). The Cochrane review, published in 2010, assessed the effectiveness of sucrose in randomized controlled trials in neonates, found 44 published studies that included 3500 infants with gestational ages from 25 to 42 weeks (Stevens *et al.*, 2010). Sucrose was shown to reduce behavioural indicators and composite pain scores upon heel lancing, venipuncture and subcutaneous injections. Significant decreases in crying, grimacing, heart rate, and pain scores were reported in neonates who were

given sucrose solution before a procedure when compared with water, and repeating the dose every 2 minutes up to 3 times increased the effect.

Sucrose is one of the most commonly studied non-pharmacological interventions for the relief of procedural pain in neonates. Many difficulties lie in carrying out neonatal clinical trials investigating safe and effective pain relief (Anand *et al.*, 2005). A significant limitation is the inability of infants to accurately convey the degree of pain relief experienced. Indirect measurement of analgesic effectiveness necessarily depend upon behavioural and physiological pain assessment scales (Ranger *et al.*, 2007); although many methods of pain assessment are available none of them are widely accepted or clearly superior to others (Duhn *et al.*, 2004). Indeed, every trial reported in the Cochrane Review of sucrose analgesia relied upon behavioural and physiological measures (Stevens *et al.*, 2010). Pain behaviour such as vocalization, facial expression and body movement are often used as indicators due to their reproducibility and ease of detection; however distinguishing behaviour due to pain perception from other forms of distress such as hunger or thirst is challenging. Furthermore, current behavioural methods necessarily rely on subjective observations. In addition, discrepancies between behavioral and physiological pain indicators have been reported (Ranger *et al.*, 2007), and together with the subjective measurement of observations suggest that current pain assessment technique may not be reflective of a reduction in the activation of central pathways to provide analgesia.

The cutaneous withdrawal reflex has been used to assess the effect of local anaesthetics in the neonate. Topical amethocaine gel and EMLA cream have been shown to reduce cutaneous reflex sensitivity to mechanical stimulation of the infant foot (Fitzgerald *et al.*, 1989; Jain *et al.*, 2000a). However, quantitative assessment of cutaneous withdrawal reflex activity in these studies was limited by the reliance on behavioural observations to measure of limb movement. Direct recordings of flexion withdrawal reflex EMG activity have not been incorporated into any randomised-controlled trials investigating anaesthetic or analgesic effectiveness, including sucrose, in the neonate. The results of Study 1 in this thesis provide a basis for quantitative measurement of flexion withdrawal reflex activity using surface EMG recordings. This technique has many advantages, it is non-invasive and provides minimal discomfort to the infant, and importantly provides a quantitative indication of the level of nociceptive transmission in the spinal cord.

5.2 Aim of the chapter

The aim of this chapter was to investigate the effect of a standard, clinically used analgesic on the nociceptive flexion withdrawal reflex in full-term infants. This chapter reports on the effect of oral sucrose administration prior to a noxious heel lance compared to sterile water (control) using surface EMG recordings of flexion withdrawal reflex activity from the biceps femoris, as an outcome measure. For comparison, clinical pain assessment scores were also recorded. This study was part of a double-blind randomised controlled trial to test the analgesic efficacy of sucrose using a number of outcome measures (Slater et al., 2010a).

The key objectives were:

(1) To characterise and compare the effect of sucrose and sterile water on the properties of noxious-evoked flexion withdrawal reflex activity in the full-term infant

(2) To test whether ipsi- and contralateral nociceptive flexion withdrawal reflex activity are differentially altered by sucrose treatment

(3) To assess the effect of sucrose on clinical pain assessment scores and their relationship with flexion withdrawal reflex activity

5.3 Methods

5.3.1 Organisation of a randomised controlled trial

Designing a randomised controlled trial requires a number of issues to be considered including (1) adherence to international standards, (2) prevention of experimental bias, and (3) medical care of the patient (in addition to those discussed in General Methods).

(1) Adherence to international standards

The Medicines and Healthcare products Regulatory Agency (MHRA) advised that this trial should be designed on the basis that sucrose was classified as '*a non-clinical trial of an investigational medicinal product (non-CTIMP)'*. The classification was based on sucrose being a food product and that the purpose of the clinical trial did not involve investigating the safety of a product.

The clinical trial conformed to the *ICH Harmonised Tripartite Guidelines* for *Good Clinical Practice* (1996) and the *Consolidated Standards of Reporting Trials (CONSORT) (Schulz et al.)*. Clinical trial registration with the International Standard Randomised Controlled Trial Number Register was obtained; Trial ID: *ISRCTN78390996* (http://www.controlled-trials.com/ISRCTN78390996).

(2) Prevention of experimental bias

The design of the clinical trial was rigorous to prevent experimental bias. Accordingly, infants were assigned either sucrose or sterile-water (control) using randomised allocation. Each solution was given a four-character identification number that did not allude to the treatment identity. The researchers and clinical staff remained blinded to the treatment identity for the duration of the clinical trial.

(3) Medical care of the patient

Sucrose is not a standard intervention for pain-relief at UCH. Furthermore, acute noxious procedures such as a heel lance for blood sampling are performed in the absence of analgesia in accordance with UCH guidelines. Participation in the clinical trial did not intervene with the clinical care of the infant nor deny analgesic medication for the purpose of this research.

For these reasons, it was justified to randomise in-patients at UCH to receive either sucrose or sterile water treatment prior to an acute noxious stimulus (a clinically required heel lance).

Adverse effects of sucrose use in the neonate have not been reported. In the event of an adverse effect, the hospital pharmacist had access to a numbered list containing treatment identities of each solution administered and could inform clinicians of the exact treatment a patient received if required. At no point during the trial was medical treatment withheld from patients or their medical care hindered.

5.3.2 Participants

All participants were in-patients on the postnatal ward at UCH aged between 37 and 43 weeks GA (full-term infants). Participants were included in the study if they conformed to the criteria outlined in the General Methods (see section 2.2). Infants were additionally ineligible for inclusion if they were (1) asleep, (2) fed <30 minutes before the heel lance, (3) born to mothers who were diabetic, or (4) had previous surgery In addition, infants were excluded from the study if they had contraindications to the administration of sucrose: (1) high risk for necrotising enterocolitis, (2) asphyxiated infants, (3) feeding intolerance, (4) absent bowel sounds, (5) oesophageal atresia or tracheal oesophageal fistula, or (6) active phase persistent pulmonary hypertension of the newborn.

Fifty-five studies were conducted with EMG recordings; individual characteristics are described below (Table 5-1).

Infant No.	Sex	Multiple births	GA at birth (weeks)	GA at study (weeks)	PNA (days)	Weight at birth (g)	Weight at study (g)	Reason for admission
1	Female	Singleton	40.43	41.29	6	4000	4000	Establishing feeds
2	Male	Singleton	40.14	40.43	2	3480	3480	Jaundice
3	Female	Singleton	39.29	39.43	1	2770	2770	Establishing feeds
4	Female	Singleton	39.14	39.29	1	3570	3570	Jaundice
5	Male	Singleton	38.57	38.86	2	3880	3880	Jaundice
6	Female	Singleton	39.86	41.00	8	3300	2952	Monitoring blood sugar level
7	Female	Singleton	40.86	41.14	2	3560	3560	Jaundice
8	Male	Singleton	41.57	42.00	3	4318	4318	Infection
9	Female	Singleton	41.14	41.43	2	3370	3370	Jaundice
10	Male	Singleton	38.43	39.14	5	2260	2142	Infection
11	Male	Singleton	40.57	40.86	2	3690	3595	Jaundice
12	Male	Singleton	38.14	39.00	6	3448	3395	Jaundice

Infant No.	Sex	Multiple births	GA at birth (weeks)	GA at study (weeks)	PNA (days)	Weight at birth (g)	Weight at study (g)	Reason for admission
13	Male	Singleton	39.00	39.57	4	2820	2590	Jaundice
14	Male	Singleton	41.57	42.00	3	4110	4110	Infection
15	Female	Singleton	38.29	38.57	2	3100	3100	Infection
16	Female	Singleton	39.00	39.43	3	3340	3340	Jaundice
17	Male	Singleton	39.00	39.29	2	2860	2860	Infection
18	Female	Singleton	41.14	41.43	2	3700	3700	Respiratory Distress
19	Female	Singleton	40.71	40.86	1	3470	3470	Jaundice
20	Male	Singleton	38.86	39.00	1	3158	3158	Infection
21	Female	Singleton	41.14	41.71	4	3940	3940	Jaundice
22	Female	Singleton	37.14	37.43	2	2790	2790	Jaundice
23	Male	Singleton	38.00	38.57	4	3280	3250	Infection
24	Male	Singleton	38.00	38.43	3	3140	3140	Jaundice
25	Female	Singleton	39.29	39.57	2	3316	3316	Jaundice
26	Male	Singleton	41.00	41.29	2	2980	2980	Jaundice
27	Male	Singleton	38.57	39.57	7	4250	4250	Jaundice
28	Female	Singleton	39.00	39.29	2	3390	3390	Jaundice
29	Male	Singleton	37.86	38.57	5	2840	2840	Jaundice
30	Female	Singleton	39.29	39.57	2	3560	3560	Jaundice
31	Male	Singleton	39.86	40.43	4	3100	3100	Infection
32	Male	Singleton	40.00	41.00	7	4100	3865	Jaundice / infection
33	Male	Singleton	40.14	40.43	2	4160	4160	Jaundice
34	Male	Singleton	42.00	42.14	1	3560	3560	Infection
35	Male	Singleton	39.29	40.14	6	3750	3750	Jaundice
36	Female	Singleton	39.00	39.43	3	3014	3014	Jaundice
37	Male	Singleton	39.00	39.29	2	2890	2890	Jaundice
38	Male	Twin	38.00	38.86	6	2870	2870	Jaundice
39	Male	Singleton	41.29	41.43	1	3490	3490	Infection
40	Female	Singleton	40.43	40.71	2	3630	3590	Infection
41	Female	Singleton	39.00	39.43	3	3200	2942	Jaundice
42	Male	Singleton	40.57	41.29	5	3530	3530	Infection
43	Male	Singleton	40.43	41.00	4	3140	2800	Jaundice
44	Male	Singleton	40.43	40.43	0	3490	3490	Jaundice
45	Male	Singleton	40.57	40.86	2	3000	3000	Jaundice
46	Male	Singleton	39.29	40.00	5	3030	2804	Establishing feeds
47	Male	Singleton	39.57	40.14	4	3560	3584	Establishing feeds
48	Female	Singleton	40.86	41.14	2	3630	3630	Jaundice
49	Female	Singleton	41.43	41.57	1	3290	3290	Jaundice
50	Female	Singleton	41.71	42.43	5	3440	3440	Establishing feeds
51	Male	Singleton	39.86	40.43	4	3200	3200	Infection
52	Male	Singleton	38.43	38.71	2	3370	3370	Infection
53	Male	Singleton	41.71	42.43	5	3700	3640	Jaundice

Infant No.	Sex	Multiple births	GA at birth (weeks)	GA at study (weeks)	PNA (days)	Weight at birth (g)	Weight at study (g)	Reason for admission
54	Female	Singleton	38.71	39.14	3	3720	3414	Jaundice
55	Male	Singleton	41.57	42.14	4	3860	3860	Infection

Table 5-1: Individual characteristics of infants included in the study
GA, gestational age; PNA, postnatal age. Of the 59 studies conducted using EEG recordings, 55 of these also had EMG recordings and are detailed here (see Figure 5-3: Flow of participants through the trial).

5.3.3 Study design

Subjects received a 0.5ml solution of 24% sucrose or sterile water onto the tongue, two minutes prior to a clinically required heel lance procedure. The protocol for performing the stimulus was conducted as described in the General Methods (section 2.4). Spinal flexion reflex withdrawal activity, facial behaviour and physiological changes evoked by the noxious stimulus were measured using time-locked surface EMG, video and pulse oximetry recording techniques. A schematic of the experimental time-line is shown in Figure 5-1.

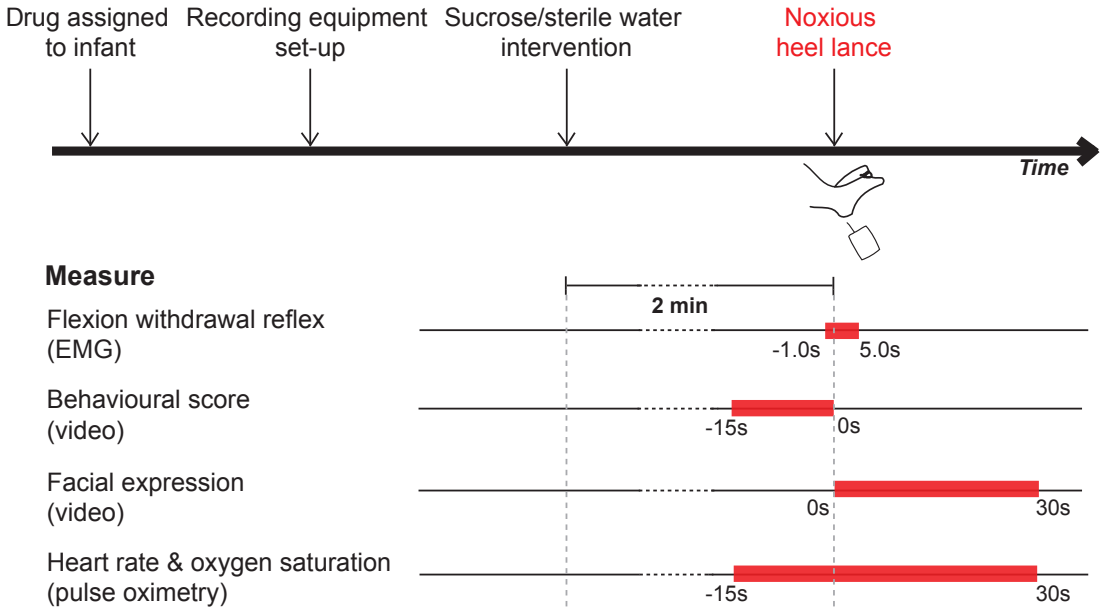


Figure 5-1: Experimental time-line
Vertical dotted lines indicate the time where sucrose/sterile water solution was administered or noxious event occurred. Coloured blocks represent the recording epoch used in EMG, video or pulse oximetry data analysis (epoch size annotated by each block). Adapted from Slater et al (2010).

5.3.3.1 Summary of treatments

Subjects were allocated at random to receive one of two solutions: 24% sucrose or sterile water. There was a 50 % chance of receiving sucrose. A clinician prescribed the treatment solution, and 0.5ml was administered directly onto the anterior surface of the tongue using a 1ml syringe two minutes prior a noxious heel lance. The dosage, route of administration and time course is recommended in the guidelines for use of 24 % sucrose (Stevens *et al.*, 2010). To adhere to current clinical practice, the dose and expiry date of each solution was double-checked by two neonatal practitioners and a neonatal nurse administered all solutions.

5.3.3.2 Randomisation and blinding

Individuals were randomised to one of two treatment groups using computer-generated block-randomisation. Sixty samples were included in the randomised controlled trial. There were six blocks of ten samples with equal allocation of treatment arms; each block contained five samples of sucrose and five samples of sterile water.

The treatment randomisation was performed by the UCH pharmacy. The pharmacy received the sterile sucrose solution (24% sucrose in purified water) and purified water sample from Inspiration Healthcare (Respironics Inc., USA), in individual 15ml vials, which could not be visually distinguished by the packaging or by colour, smell and flow during administration.

The pharmacy labelled each vial with a randomisation code that corresponded to the identity of the solutions. The number on the vial was recorded with (1) the patient's hospital prescription chart for hospital purposes and (2) patient's data for research purposes. Only the hospital pharmacy had access to the randomisation codes that could be used to identify the solution. A sealed copy of the randomisation chart was also stored in the neonatal unit in case an adverse event was reported. The identification of the treatments remained anonymous to the research team until the end of the study.

5.3.3.3 Breaking of the randomisation code

When the study officially ended and the data analysis complete, the sample identification list was released to the researchers so that the treatment groups could be correctly identified. Throughout the study the researchers, clinicians, subjects and parents were blinded to the

identity of the solutions. Inspiration Healthcare was not involved in the study design, data collection or data analysis. No interim analysis was performed in this study.

5.3.3.4 Adverse effects

Oral administration of 0.5ml of 24% sucrose or sterile water does not have any adverse effects in the neonate. There were no potential adverse effects, risks or hazards for research participants from giving sucrose or from other interventions during the study. In the event of an adverse effect, the event was recorded in the hospital notes, patient information sheet specific to the clinical trial and the Chief Investigator notified.

5.3.4 Recording

The protocol for EMG and video recording is described in the General Methods (section 2.6). Physiological recordings of oxygen saturation and heart rate were simultaneously measured. A transcutaneous pulse oximeter probe (Nellcor N-560) was positioned on the foot contralateral to the site of stimulation. Pulse oximetry data were electronically linked to the computer and were recorded for the duration of the study. The time of noxious stimulus application was automatically event-marked on the recording by electronically linking the lancet device to the recording equipment (section 2.5).

Fifty-five studies were conducted with ipsilateral EMG recordings of biceps femoris activity, video recordings of facial expression, and pulse oximetry for heart rate and oxygen saturation data acquisition. Of these, 48 studies had contralateral EMG recordings.

5.3.5 Data analysis

Infants were separated into two groups depending on the treatment assigned: 'sucrose' and 'sterile-water' treated infants.

5.3.5.1 EMG analysis

EMG analysis is described in detail in the General Methods (see section 2.7.1 on page 70). The approach used to quantify flexion withdrawal reflex activity in Study 1 of this thesis was adopted for this data. A clear reflex response to a noxious heel lance was defined as a change in EMG activity that exceeded 3SD of baseline activity. Baseline activity (-1000ms to time 0)

was stable and there were no significant differences in mean baseline activity between sucrose and sterile water treatment groups. The reflex EMG response was quantified by:

(1) Latency: *Onset latency, limited to the first 2000ms after the stimulus (ms) and Peak latency (ms)*

(2) The pattern of activity: *(i) Non-latency corrected & (ii) Latency-corrected analysis*

(3) Mean amplitude: *(i) Non-latency corrected & (ii) Latency-corrected analysis*

5.3.5.2 Clinical pain assessment (PIPP)

Clinical pain assessment was scored using the PIPP (see section 2.7.2). The PIPP is validated technique integrating age, behaviour and physiological measures to give a numerical score [Table 2-2 on page 74] (Ballantyne *et al.*, 1999; Stevens *et al.*, 1996). The emotional 'pain experience' theoretically occurs where the PIPP exceeds a score of 6; the maximum possible PIPP score was 21.

The behavioural components of the PIPP score were analysed using the methods described in (see section 2.7.2 on page 73; Table 2-2). A facial response was characterised by the presence brow bulge, eye squeeze and nasolabial furrow behavioural features. Figure 5-2 illustrates the fundamental time-points in the study and associated facial response in an example infant.

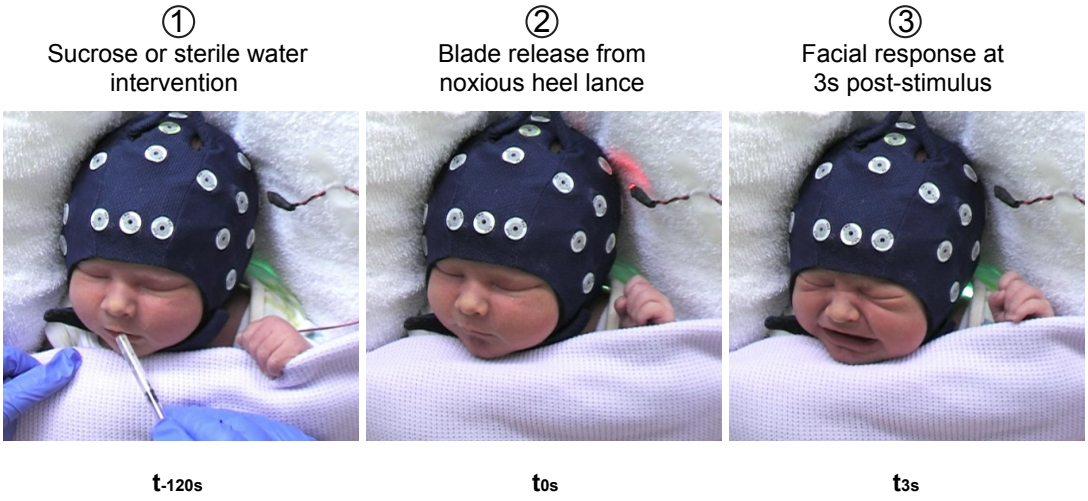


Figure 5-2: Example infant at fundamental time-points in the study
(1) Sucrose or sterile water administration 2 minutes prior to heel lance procedure (t_{120s}); (2) time of stimulus (identified by LED flash), (t_{0s}); and (3) facial expression at 3 seconds post-stimulus (t_{3s}), all 3 key facial expression features (brow bulge, eye squeeze and nasolabial furrow) are exhibited.

Maximum change in heart rate (bpm) and minimum change in oxygen saturation (%) from mean baseline activity (15s before the stimulus) were measured using pulse oximetry and individually scored.

In a series of analyses independent of the PIPP score, the evoked facial behaviour was quantified using the measures below:

(1) Incidence: *number of infants expressing a visible facial response (n/N; %)*

(2) Latency: *time to first visible facial response (s)*

(3) TFS: The duration of brow bulge, eye-squeeze and nasolabial furrow features were individually scored and combined to give a total facial score

5.3.6 Statistical analysis

Significance testing was performed as follows:

(1) Latency to onset of reflex response, and peak activity, were compared using a *Student's t-test* for (1) treatment differences – sucrose versus sterile water (unpaired t-test), (2) laterality -ipsilateral versus contralateral activity (paired t-test), and (3) the association with a visible facial response– TFS=0 versus TFS>0 (unpaired t-test).

(2) The pattern of flexion withdrawal reflex activity was compared for (1) treatment differences - sucrose versus sterile-water, (2) laterality –ipsilateral versus contralateral biceps femoris activity, and (3) the association with a visible facial response –infants with a TFS=0 versus TFS>0, using a *two-way ANOVA with repeated measures and Bonferroni post-hoc* testing over the recording time period.

(3) The mean activity over 2000ms was compared using a *Student's t-test* for (1) treatment differences – sucrose versus sterile water (unpaired t-test) and (2) laterality - ipsilateral versus contralateral activity (paired t-test), (3) the association with a visible facial response –a TFS=0 versus TFS>0 (unpaired t-test).

(4) Total PIPP scores and individual PIPP components were compared for sucrose versus sterile water using a *Student's unpaired t-test.*

(5) Facial behaviour was compared for (1) latency to first visible facial response between treatment groups using a *Student's unpaired t-test.* (2) The association between treatment groups, incidences of flexion withdrawal reflex activity and a visible facial response was tested using *Fisher's exact test.*

5.4 Results

Fifty-nine infants were randomised to receive sucrose or sterile-water, of which 55 had EMG recordings to assess flexion withdrawal reflex activity. 34 EMG recordings were included in the final trial analysis. Figure 5-3 is a flow diagram of participants through the trial and shows the rationale for excluding some EMG recordings from the final analysis.

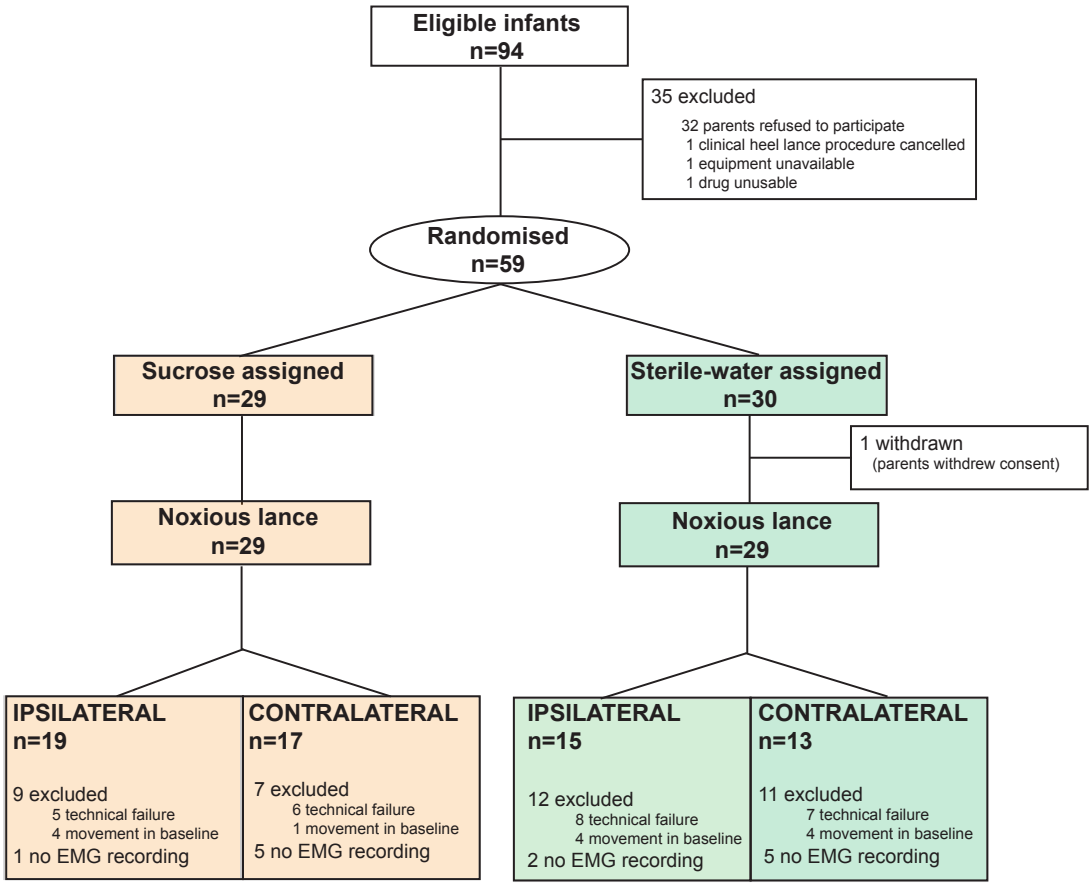


Figure 5-3: Flow of participants through the trial
All infants had EEG recordings (the primary measure investigated the effect of oral sucrose on the morphology of the nociceptive-specific potential at the vertex) and the majority also had ipsilateral EMG recordings (n=55). Reasons for no EMG recording were due to the immediate necessity to obtain the clinical blood sample or instability of the infant(s) in tolerating additional handling. EMG recordings were excluded from the analysis for technical failure and movement in the baseline using criteria described in the General Methods.

To begin with, the properties of the ipsilateral flexion withdrawal reflex EMG activity were examined in sucrose and sterile-water treated infants. The demographics for each treatment group are summarised in Table 5-2.

	Sucrose (N=19)	Sterile-water (N=15)
Male; (n/N)	47%; (9/19)	60%; 9/15
Mean GA at birth (weeks)	39.57±1.06; range 38.00-41.43	39.82±1.39; range 37.14-42.00
Mean GA at study (weeks)	39.99±1.02; range 38.57-41.57	40.20±1.29; range 37.43-42.14
Mean PNA (days)	2.95±2.09; range 1-8	2.67±1.68; range 0-6
Mean weight at study (g)	3331.42±407.08; range 2770.0-4250.0	3319.53±348.89; range 2790.0-3570.0
Right heel stimulated; (n/N)	74%; 14/19	40%; 6/15

Table 5-2: Infant demographics of each treatment group
Data are expressed as mean ±standard deviation unless otherwise stated.

5.4.1 The effect of sterile-water 'control' on nociceptive flexion withdrawal reflex EMG activity

Flexion withdrawal reflex activity was initially characterised in sterile-water treated infants (n=15). Example recordings are shown in Figure 5-4 on page 186. This was the control group for the trial and the aim here was to establish the characteristics of the EMG activity in this group for comparison with the 'naive' controls described in Chapter 2, Study 1.

5.4.1.1 Latency

All sterile-water treated infants exhibited a clear flexion withdrawal reflex response. The mean latency to onset of flexion withdrawal reflex activity was 411.6ms (95% CI 303.4-519.7ms). The mean peak latency was 983.3ms (95% CI 719.3-1247ms). The variation in absolute latency values for the two measures is shown in Figure 5-9 on page 192.

5.4.1.2 Pattern of reflex activity: (1) non-latency corrected

The pattern of activity was quantified in real time using non-latency corrected analysis. The RMS of 250ms time bins was calculated for each infant to identify how the pattern of flexion withdrawal changed in magnitude over time. The pattern of activity for individual infants is shown in Figure 5-5.

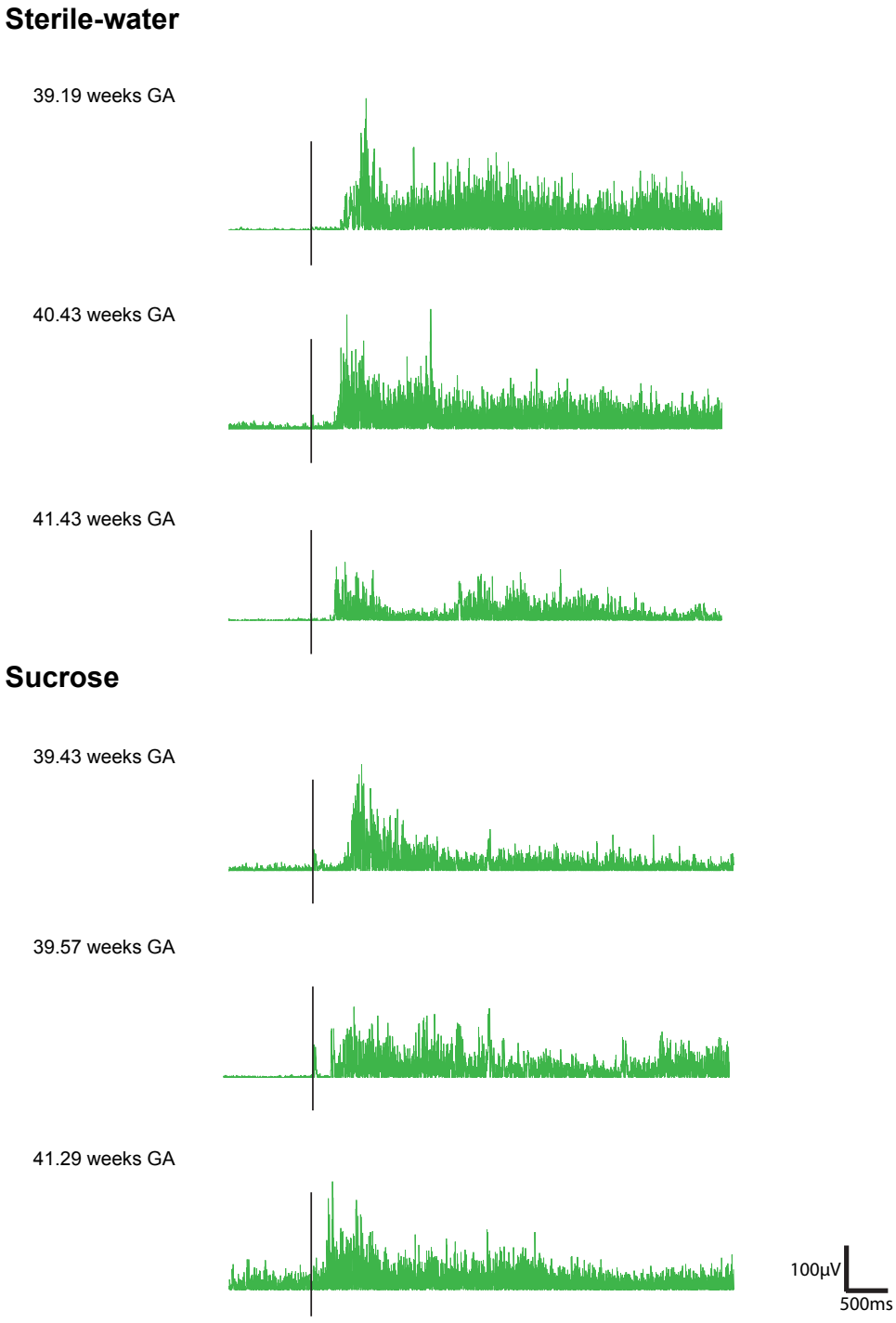


Figure 5-4: Example EMG recordings from sterile-water and sucrose treated infants following a noxious heel lance
Vertical line indicates the time of noxious heel lance. Scale bar is given in the bottom right corner.

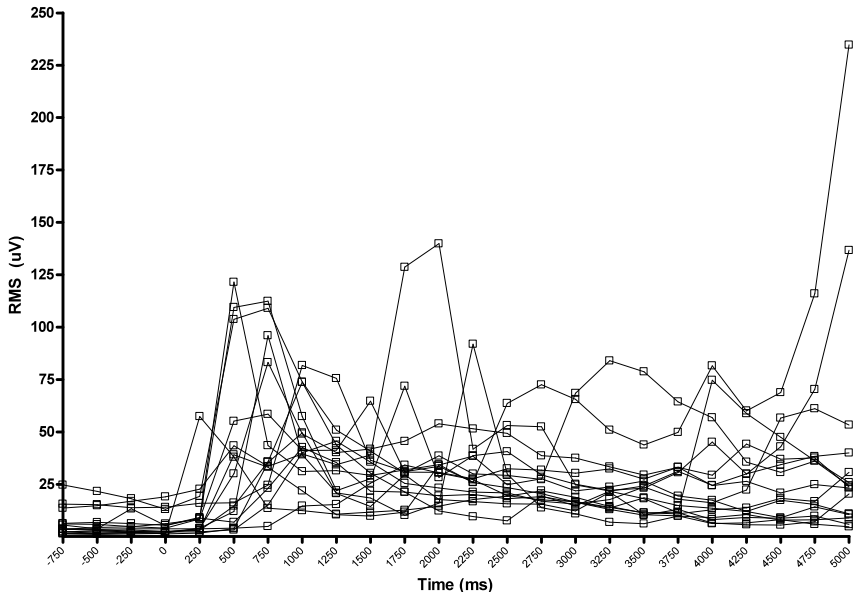


Figure 5-5: Individual ipsilateral bipces femoris activity of sterile-water treated infants (n=15) following a noxious heel lance
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins i.e. -750 = activity between -1000 and -750ms. The noxious stimulus was applied at time 0.

The mean pattern of activity for the sterile-water treated group is shown in Figure 5-6. Flexion withdrawal reflex EMG activity rapidly increased in the first 750ms after the stimulus. Mean peak activity was 48.10μV (95% CI 28.48-67.72μV) and was between 500ms and 750ms after the stimulus. Activity subsequently decreased until 1250-1500ms where a second wave of activity of smaller magnitude occurred. Over the post-stimulus period, evoked activity remained raised and did not decrease to baseline levels.

5.4.1.3 Pattern of reflex activity (2) latency-corrected

The same EMG recordings were analysed using latency-corrected analysis, a method that corrected for variability in onset latency. This method permits the characteristics of actual reflex activity to be determined and is useful in testing treatment effects later in this chapter. Latency-corrected analysis of the group mean is shown in Figure 5-11 on page 193. Corrected peak activity was 59.08μV (95% CI 37.91-80.26μV) and occurred in the first 250ms of the reflex response. Flexion withdrawal reflex activity subsequently decreased over the following 750ms before a second, small increase in activity occurred. Evoked activity did not return to baseline levels throughout the recording epoch period.

5.4.1.4 Comparison between flexion withdrawal reflex activity in sterile-water treated infants versus naive infants at full-term

To test the effect of sterile-water administration on the typical characteristics of noxious-evoked flexion withdrawal reflex activity, a comparison was made against the sample of naive full-term infants (n=19) analysed previously in this thesis (see Study 1). The latter group did not receive any oral intervention prior to the noxious heel lance and were classed as 'naïve'.

Sterile-water intervention had no effect on flexion withdrawal reflex properties. The key measures used to characterise EMG activity -latency to onset and peak activity, and pattern of activity were compared; no significant differences were found in any measure (Table 5-3).

	Sterile-water (N=15)	Naïve (N=19)	p-value
Mean baseline activity (μV) [1]	6.24 (2.98-9.50)	5.10 (3.29-6.89)	0.49
Latency to response (ms) [1]	411.6 (303.4-519.7)	369.1 (277.3-460.9)	0.49
Latency to peak activity (ms) [1]	983.3 (719.3-1247.0)	934.2 (742.2-1126.0)	0.92
Mean activity over 2000ms (μV) [1]	44.50 (34.90-54.10)	37.99 (29.76-46.21)	0.28

Table 5-3: Summary of ipsilateral flexion withdrawal reflex activity in sterile-water treated infants and naive infants following a noxious heel lance
All data expressed as mean (95% CI). [1]Students unpaired t-test. [2]Two-way ANOVA with repeated measures.

Similarities in the pattern of flexion withdrawal reflex activity are illustrated on page 189 (Non-latency corrected, Figure 5-6; latency corrected, Figure 5-7). Accounting for variation in latency to onset of the reflex response made no difference to the pattern of reflex response. Mean activity over 2000ms from the onset of reflex activity (latency-corrected) was calculated to summarise the magnitude of reflex activity, no significant differences were found (unpaired t-test, p=0.28; Table 5-3).

These results indicate that sterile-water is a suitable control group for comparison against the effects of sucrose on the flexion withdrawal reflex properties.

Non-latency corrected

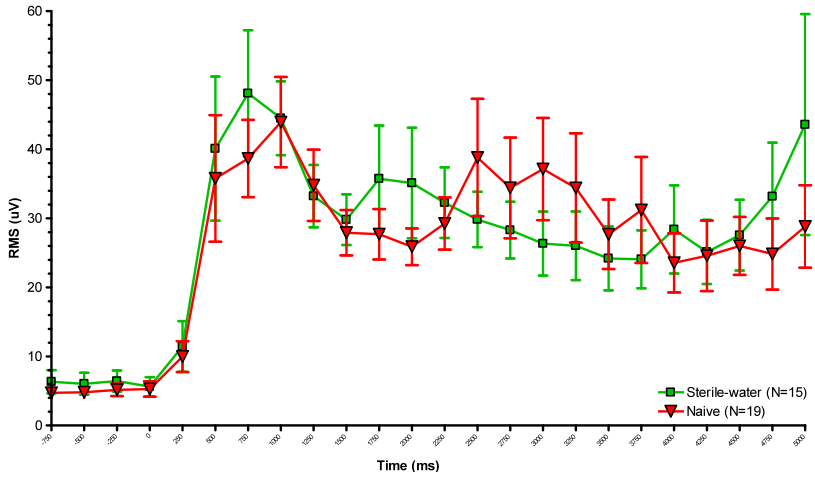


Figure 5-6: No difference in the pattern of flexion withdrawal reflex activity in sterile-water treated infants verses naive infants following a noxious heel lance

There were no differences in evoked EMG reflex activity between sterile-water and naïve infants ($F_{1,736}$=0.07; p=0.80). The RMS±(standard error) of each time bin is shown i.e. -750 = activity between -1000ms and -750ms.

Latency corrected

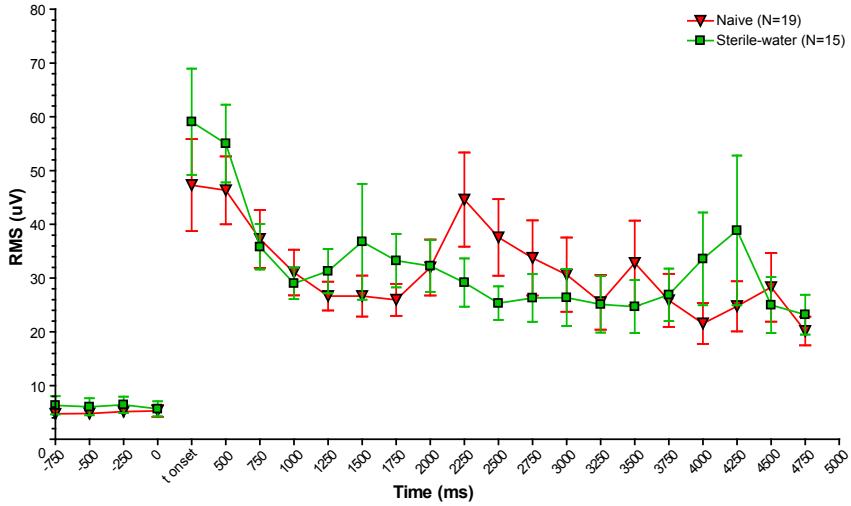


Figure 5-7: No difference in the pattern of flexion withdrawal reflex activity in sterile-water treated infants verses naive infants following a noxious heel lance

There were no differences in evoked EMG reflex activity between sterile-water and naïve infants ($F_{1,704}$=0.06; p=0.81). Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recording epoch (in completed 250ms time bins). The RMS (±standard error) in each time bin is displayed.

5.4.2 The effect of sucrose on nociceptive flexion withdrawal reflex EMG activity

Flexion withdrawal reflex activity was subsequently characterised in sucrose-treated infants (n=19). Example recordings from sucrose-treated infants are shown in Figure 5-4 on page 186.

5.4.2.1 Latency

A clear flexion withdrawal reflex response was observed in 18/19 infants. The latency to onset of flexion withdrawal reflex activity was 362.0ms (95% CI 259.4-464.6ms). The mean time taken to peak amplitude was 1013.0ms (95% CI 818.6-1208.0ms). The variation in absolute latency values for the two measures is shown in Figure 5-9 on page 179.

5.4.2.2 Pattern of reflex activity: (1) non-latency corrected

The pattern of activity for individual infants (Figure 5-8) and group average (Figure 5-11 on page 193) are shown when non-latency corrected. For the group average, the pattern of activity rapidly increased over the first 750ms after the noxious stimulus. Mean peak activity was 46.66μV (95% CI 29.21-64.11μV) between 500ms and 750ms after the stimulus. Subsequent activity decreased until 1250-1500ms where it remained at a sustained level for the duration of the remaining recording period. Activity at 1750-2000ms had decreased by 49% from mean peak activity, measuring 23.68μV (95% CI 16.42-30.95μV). Evoked activity did not return to baseline levels over the 5s post-stimulus recording window.

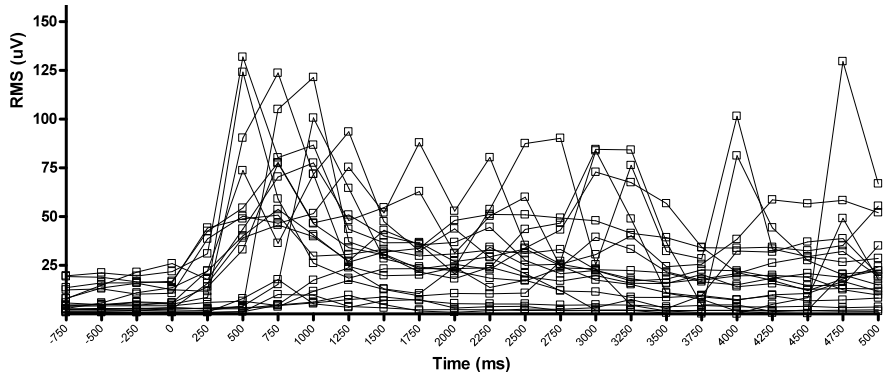


Figure 5-8: Individual ipsilateral biceps femoris activity of sucrose-treated infants (n=19) following a noxious heel lance
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins. The noxious stimulus was applied at time 0.

5.4.2.3 Pattern of reflex activity: (2) latency-corrected

Latency-corrected analysis was performed on the same EMG recordings to correct for variability in onset latency. The group mean is shown in Figure 5-11 on page 193. Corrected peak activity was 58.68μV (95% CI 34.95-82.42μV) and occurred in the first 250ms of the reflex response. Flexion withdrawal reflex activity subsequently decreased over the following 750ms before a second, small increase in activity occurred. Evoked activity did not return to baseline levels throughout the recording epoch period.

5.4.3 Comparison of flexion withdrawal reflex activity in sucrose versus sterile-water treated infants

Following characterisation of flexion withdrawal reflex EMG activity for sterile-water and sucrose treatment prior to a noxious heel lance, the evoked activity was compared between treatment groups; sucrose (n=19); sterile-water (n=15).

5.4.3.1 Sucrose had no effect on latency properties

Sucrose-treatment did not significantly affect latency to onset of flexion withdrawal reflex activity (unpaired t-test, p=0.49). Onset latency in sterile-water treated infants was 411.6ms (95% CI 303.4-519.7ms) and in sucrose-treated infants was 362.0ms (95% CI 259.4-464.6ms) [Figure 5-9]. In addition, sucrose-treatment had no effect on the time taken to reach peak amplitude (unpaired t-test, p=0.84). Infants assigned to sterile-water exhibited a mean peak latency of 983.3ms (719.3-1247.0ms) and those assigned to sucrose exhibited a mean peak latency of 1013.0ms (95% CI 818.6-1208.0ms); [Figure 5-9].

5.4.3.2 Sucrose had no effect on the pattern of activity

The previous sections characterised reflex EMG activity using both non-latency and latency-corrected analysis. Here, the appropriate analysis to compare the effect of sucrose treatment on the time course of reflex EMG activity was latency-corrected analysis. This method has the advantage of accounting for variability in onset latency and better represents evoked reflex activity. The pattern of activity when non-latency corrected are shown for illustrative purposes (Figure 5-11; $F_{1,736}$=0.72 p=0.40).

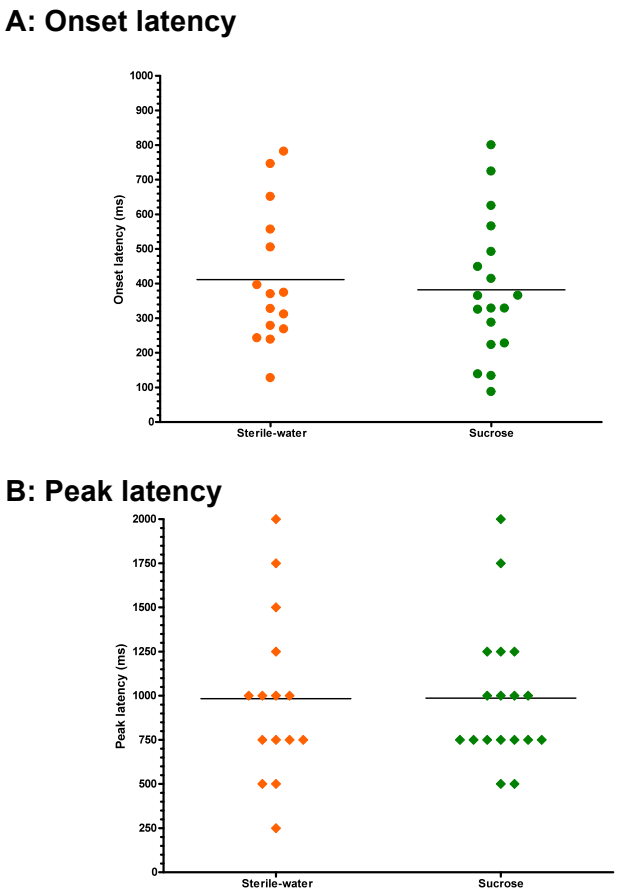


Figure 5-9 Sucrose treatment does not alter (A) onset latency or (B) peak activity of flexion withdrawal reflex activity
Each dot represents an individual infant; sucrose (n=18), sterile-water (n=15). One infant was given sucrose prior to a heel lance and did not exhibit detectable reflex response/ onset latency.

The pattern of flexion withdrawal reflex activity when latency-corrected is shown in Figure 5-11 on page 193. Sucrose had no effect on the pattern of flexion withdrawal reflex activity compared to sterile-water ($F_{1, 682}$=0.50; p=0.48). Peak activity occurred in the first 250ms of the evoked reflex response and rapidly decreased over the following 750ms for both treatment groups. Furthermore, evoked activity did not return to baseline levels over the duration of the recording period.

The mean (RMS) activity over 2000ms from onset of reflex activity (latency-corrected) was determined to summarise flexion withdrawal reflex activity. The magnitude of EMG activity was equivalent for the sucrose, 40.78μV (95% CI 30.12-51.44μV) and sterile-water, 44.50μV (95% CI 34.90-54.10μV) treated groups; unpaired t-test, p=0.59.

Non-latency corrected

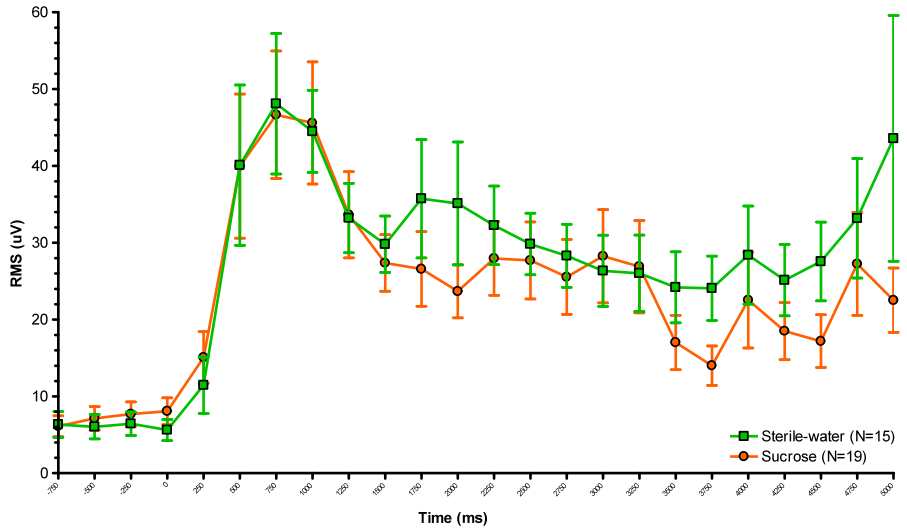


Figure 5-10: Sucrose-treatment does not reduce flexion withdrawal reflex activity following a noxious heel lance (non-latency corrected analysis)
The RMS (±standard error) of each time bin is shown i.e.-750 = activity between -1000ms and -750ms.

Latency corrected

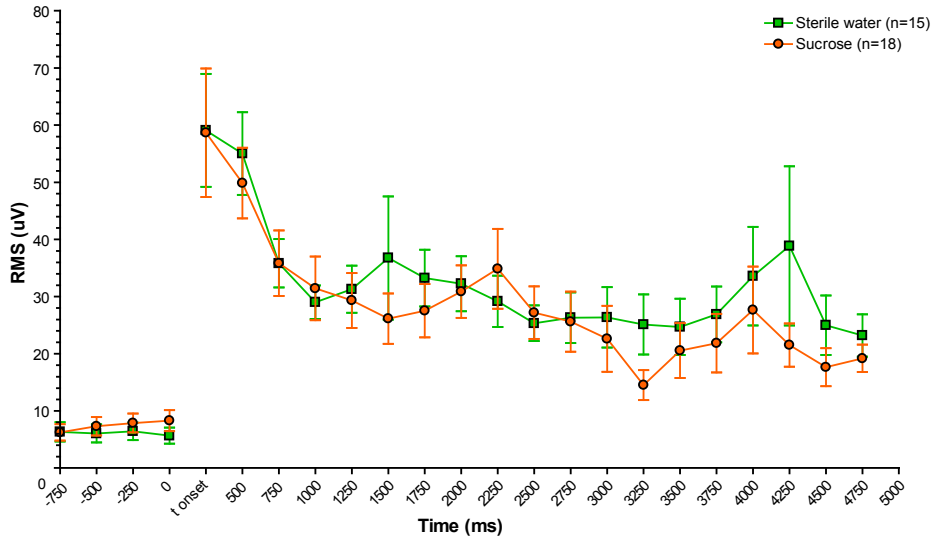


Figure 5-11: Sucrose-treatment does not reduce flexion withdrawal reflex activity following a noxious heel lance (latency-corrected analysis)
Ipsilateral biceps femoris activity was calculated using the RMS of 250ms time bins between -1000ms and 0 (time of stimulus), and between the time of onset of a reflex response (t onset) and the end of the recording epoch (in completed 250ms time bins). The RMS (±standard error) in each time bin is displayed.

Summary 1

This section of work characterised noxious-evoked flexion withdrawal reflex activity in full-term infants following oral sucrose and sterile-water administration. A summary of the flexion withdrawal reflex properties for sucrose and sterile-water treated infants, and the associated statistical comparisons are in Table 5-4.

	Sucrose (N=19)	Sterile-water (N=15)	p-value
Mean baseline activity (μV) [1]	6.24 (2.98-9.50)	7.41 (4.19-10.63)	0.60
Latency to response (ms) [1]	411.6 (303.4-519.7)	362.0 (259.4-464.6)	0.49
Latency to peak activity (ms) [1]	983.3 (719.3-1247.0)	1013.0 (818.6-1208.0)	0.84
Mean activity over 2000ms (μV) [1]	40.78 (30.12-51.44)	44.50 (34.90-54.10)	0.43

Table 5-4: Summary of ipsilateral flexion withdrawal reflex activity following a noxious heel lance in sucrose and sterile-water treated infants
All data expressed as mean (95% CI). [1]Students unpaired t-test.

The key findings are:

- Sterile-water had no effect on ipsilateral flexion withdrawal reflex EMG activity when compared to naïve infants
- Oral sucrose (0.5ml of 24% sucrose at 2 minutes prior to a noxious heel lance) had no significant effect on latency, pattern of activity or magnitude of ipsilateral flexion withdrawal reflex activity in full-term infants when compared to sterile water controls
- Oral sucrose is ineffective in modulating ipsilateral spinal nociceptive reflexes

5.4.4 The effect of sucrose on contralateral EMG activity

In the previous chapter (Study 1), flexion withdrawal reflex activity was measured in the contralateral limb and found to exhibit comparable activity to ipsilateral flexion following a noxious heel lance. Furthermore, the degree of contralateral limb flexion was dependent on the stimulus intensity. In this section, reflex EMG properties in the contralateral biceps femoris were investigated and comparisons made between sterile-water (n=13) and sucrose (n=17) treated infants. Infant demographics of EMG recordings included in the analysis are in Table 5-5.

	Sucrose (N=17)	Sterile-water (N=13)
Male; (n/N)	62%; 8/13	56%; 5/9
Mean GA at birth (weeks)	39.46±1.15; range 38.00-41.43	40.03±1.59; range 37.14-42.00
Mean GA at study (weeks)	39.91±1.05; range 38.57-41.57	40.35±1.52; range 37.43-42.14
Mean PNA (days)	3.15±1.82; range 1-7	2.22±1.56; range 0-5
Mean weight at study (g)	3358.23±436.45; range 2860.00-4250.00	3236.00±360.96; range 2790.00-3700.00
Right heel stimulated; (n/N)	69%; 9/13	33%; 3/9

Table 5-5: Infant demographics for contralateral EMG activity in sucrose and sterile-water treated infants.
Data are expressed as mean ±standard deviation unless otherwise stated.

5.4.4.1 Sucrose has no effect on contralateral reflex latency

Contralateral flexion withdrawal reflex activity was evoked in all sterile-water (n=13) and sucrose-treated (n=17) infants. Latency to onset of contralateral flexion withdrawal reflex activity was not significantly different between treatment groups (unpaired t-test, p=0.96); sterile-water treated infants had a mean onset latency of 400.8ms (95% CI 312.7-488.9ms) and sucrose-treated infants responded at 397.9ms (95% CI 301.4-494.5ms).

The time taken to peak amplitude was not significantly different for treatment groups (unpaired t-test, p=0.18); 1154.0ms (95% CI 918.7-1389.0ms) for sterile-water infants and 941.2ms (95% CI 716.1-1166.0ms) for sucrose-treated infants.

5.4.4.2 Sucrose has no effect on the pattern of contralateral reflex activity

The pattern of activity was characterised using latency-corrected analysis and the evoked reflex response was compared between treatment groups. Sucrose treatment had no effect on contralateral biceps femoris activity when compared against sterile-water treated infants ($F_{1,616}$=0.58; p=0.45). For sterile-water treated infants, the peak activity of 54.32μV (95% CI 29.37-79.27μV) occurred within the first 500ms of the reflex response. Sucrose-treated infants exhibited peak activity of 51.20μV (95% CI 32.13-70.26μV) and this occurred within the first 250ms of the reflex response (Figure 5-12). Post-peak activity the evoked response steadily decreased but was maintained above baseline levels throughout the recording period in both treatment groups.

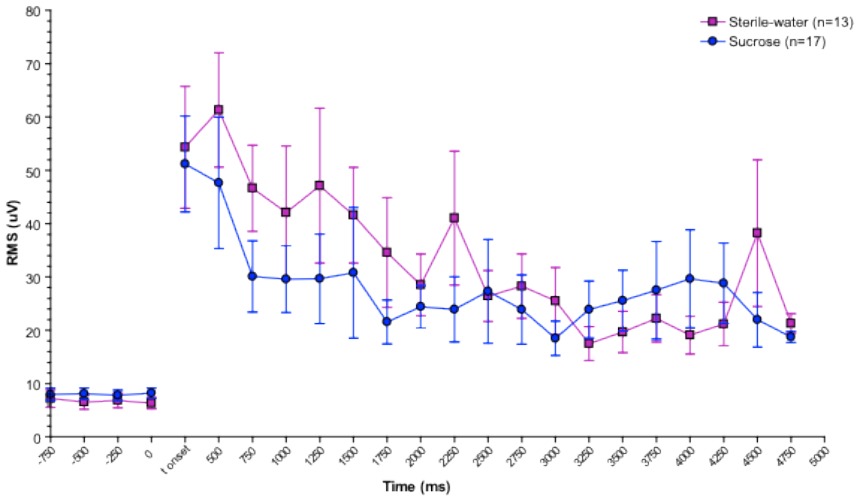


Figure 5-12: Sucrose-treatment had no effect on contralateral biceps femoris activity following a noxious heel lance (latency-corrected analysis)
The RMS±(Standard error) of each time bin is shown i.e. -750 = activity between -1000ms and -750ms.

Sucrose-treatment had no effect on the mean activity over 2000ms after the onset of reflex activity (latency-corrected), sterile-water, 50.04μV (95% CI 31.01-69.06μV, sucrose, 39.34μV (95% CI 24.52-54.15μV); unpaired t-test, p=0.34.

5.4.4.3 Bilateral flexion withdrawal reflex activity following a noxious heel lance

In a small subset of data, infants with good EMG recordings on both ipsilateral and contralateral biceps femoris were analysed following a single-noxious heel lance; sterile-water (n=9) and sucrose (n=13). This analysis was performed to assess the differences in reflex activity between limbs for each treatment group.

In all sterile-water and sucrose- treated infants, flexion withdrawal reflex activity was evoked in both the ipsilateral and contralateral biceps femoris following a noxious heel lance. There were no significant differences in the EMG reflex measures for either treatment group (Table 5-6).

The pattern of activity (latency-corrected) between ipsilateral and contralateral limbs was not significantly different in sterile-water treated infants ($F_{1,396}$=0.43; p=0.52) or in sucrose-treated infants ($F_{1,528}$=0.03; p=0.86).

(A) Sterile-water treatment (n=9)	Ipsilateral	Contralateral	p-value
Mean baseline activity (μV)	7.04 (1.91-12.16)	5.47 (2.32-8.62)	0.56
Latency to response (ms)	348.4 (228.0-468.8)	387.9 (253.5-522.3)	0.62
Latency to peak activity (ms)	944.4 (528.1-1361.0)	1111.0 (821.1-1401.0)	0.46
Mean activity over 2000ms (μV) [1]	40.89 (27.68-54.09)	50.83 (22.45-79.21)	0.75

(B) Sucrose treatment (n=13)	Ipsilateral	Contralateral	p-value
Mean baseline activity (μV)	7.30 (3.35-11.26)	7.37 (5.12-9.62)	0.46
Latency to response (ms)	306.3 (179.3-433.3)	373.5 (247.8-499.3)	0.42
Latency to peak activity (ms)	1000.0 (769.2-1231.0)	865.4 (629.8-1101.0)	0.38
Mean activity over 2000ms (μV) [1]	37.83 (24.37-51.28)	37.14 (19.35-54.93)	0.97

Table 5-6: Summary of ipsilateral and contralateral biceps femoris activity in (A) sterile-water and (B) sucrose treated infants following a noxious heel lance
All data expressed as mean (95% CI). Data analysed with Students unpaired t-test. [1]Latency-corrected analysis.

Summary 2

- Bilateral nociceptive flexion withdrawal reflex activity is recorded in sterile-water and sucrose treated infants
- EMG activity in ipsilateral and contralateral biceps femoris muscles was the same in sucrose and sterile-water treated infants
- Oral sucrose is ineffective in modulating contralateral spinal nociceptive reflexes

5.4.5 The effect of sucrose on clinical pain assessment score (PIPP): behavioural and physiological measures

Numerous randomised controlled clinical trials investigating the effectiveness of oral sucrose administration have found significant reductions in clinical pain assessment scores when compared to sterile water, a pacifier or breast-feeding (Stevens *et al.*, 2010). In this section of analysis, clinical pain scores were determined for all infants previously analysed for flexion withdrawal reflex activity (sterile-water, n=15; sucrose, n=19). Infant demographics are summarised in Table 5-2 on page 185.

5.4.5.1 Clinical pain assessment in sterile-water and sucrose treatment groups

The mean PIPP score was significantly smaller in the sucrose group, 4.84 (5% CI 2.89-6.80), compared to the sterile-water group, 8.53 (95% CI 7.37-9.70); unpaired t-test, $p<0.003$. To test differences between the behavioural and physiological components of the PIPP, each measure was compared.

(1) Baseline behaviour score (15s pre-heel lance)

The baseline behavioural score in the sucrose-treated infants was 1.21 (95% CI 0.66-1.76) and not significantly different from the sterile-water group: 1.73 (95% CI 0.99-2.47); unpaired t-test, $p= 0.23$.

(2) TFS (30s post-heel lance)

A clear change in facial motor activity was observed in every infant assigned sterile-water treatment prior to a noxious heel lance. However, in sucrose-treated infants the incidence of evoked facial activity was less frequent; 58% of infants (11/19) exhibited a TFS greater than zero.

Mean TFS in the sucrose-treated group was much lower at 2.58 (95% CI 1.19-3.97), than in the sterile-water treated group, 4.47 (95% CI 2.83-6.10); although not significantly so (unpaired t-test, $p=0.07$).

(3) Physiological score (30s post-heel lance)

Sucrose-treated infants exhibited a significantly lower physiological score of 1.05 (95% CI 0.51-1.60) than sterile-water treated infant: 2.33 (95% CI 1.53-3.14); unpaired t-test, $p=0.007$.

Heart rate and oxygen saturation comprise the physiological components of the PIPP score. The absolute values were broken down to determine if one particular factor weighted the result. Heart rate changes pre- and post- heel lance were not significantly different [sterile-water, 15.53 bpm (95% CI 9.46-21.61bpm) versus sucrose, 11.79 bpm (95% CI 3.09-20.49 bpm)]; unpaired t-test, $p=0.49$. However, the change in oxygen saturation in sucrose-treated infants was significantly smaller, 0.79% (95% CI 0.18-1.40%) than in sterile-water treated infants 2.93% (95% CI 1.43-4.44%); unpaired t-test, $p=0.005$.

5.4.5.2 Latency to first facial response

In addition to PIPP scoring, the latency to first facial response was measured. Sucrose-treated infants responded by 3.50s (95% CI 1.37-5.63s) and sterile-water treated infants by 3.67s (95% CI 0.35-6.99s) post heel lance. Five infants treated with sucrose did not exhibit an observed facial response over the 30s post-stimulus recording period. There were no significant differences in the mean latency to first observed facial response (unpaired t-test, p=0.93).

Summary 3

- Sucrose significantly lowers PIPP scores, following a heel lance, when compared to sterile-water (control) treated infants (Table 5-7).
- Baseline behavioural state is unaffected by sucrose treatment
- Sucrose treatment reduces the incidence of a detectable change in facial activity (58% compared to 0% with sterile-water)
- Sucrose treatment lowers the physiological score; autonomic function, as indicated by a minimal change in oxygen saturation, is stable during the heel lance procedure.

	Sucrose (N=19)	Sterile-water (N=15)	p-value
PIPP	4.84 (2.89-6.80)	8.53 (7.37-9.70)	**0.003**
Behavioural score	1.21 (0.66-1.76)	1.73 (0.99-2.47)	0.23
Total facial score	2.58 (1.19-3.97)	4.47 (2.83-6.10)	0.07
Physiological score	1.05 (0.51-1.60)	2.33 (1.53-3.14)	**0.007**
Incidence of facial response (n/N)	58%; 11/19	100%; 15/15	
Latency to first observed facial response (s)	3.50 (1.37-5.63)	3.67 (0.35-6.99)	0.93

Table 5-7: Clinical pain assessment in sterile-water and sucrose-treated infants following a noxious heel lance
Incidence of facial response is the percentage of infants with a total facial score greater than zero. All data expressed as mean (95% CI) unless otherwise stated. Students' unpaired t-test used for significance testing; significant values are coloured in green.

5.4.6 Comparison of the effect of sucrose on facial behaviour and flexion withdrawal reflex activity

In this section, the relationship between noxious-evoked behavioural and physiological activity was investigated using ipsilateral EMG recordings, pulse oximetry and videos that were time-locked to the stimulus. Included in the final analysis were 15 sterile-water and 19 sucrose-treated infants.

5.4.6.1 PIPP score is not correlated with flexion withdrawal reflex activity

PIPP scores for sucrose and sterile-water treated groups were compared against the magnitude of flexion withdrawal reflex activity to establish if a high PIPP score was indicative of large spinal cord excitability. Flexion withdrawal reflex activity was summarised by calculating the mean (RMS) activity over 2000ms after the stimulus. As Figure 5-13 illustrates, there was no relationship between clinical pain assessment scores and the magnitude of flexion withdrawal reflex activity for either treatment group. Correlation analysis confirmed no significant relationship between these variables for sterile-water (R^2=0.03; p=0.56) or sucrose (R^2=0.007; p=0.73).

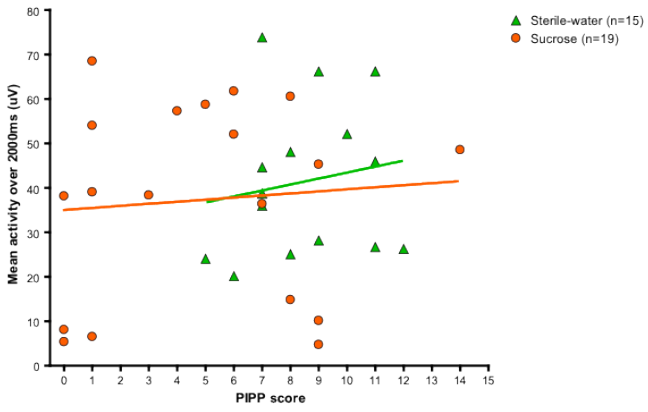


Figure 5-13: No relationship between PIPP score and magnitude of flexion withdrawal reflex activity
Correlation analysis confirmed no significant differences in sterile-water (R^2=0.03; p=0.56) and sucrose treated infants (R^2=0.007; p=0.73). Note: three infants did not have PIPP score above 0 despite the presence of flexion withdrawal reflex activity following a noxious heel lance. PIPP scores of infants treated with sucrose are distributed across the PIPP scale, with 12 infants scoring between 0 and 6. On the contrary, PIPP scores of sterile-water treated infants encompass a smaller distribution with most scores being >7. Each point represents an individual infant; green triangles represent sterile-water treatment, orange circles represent sucrose treatment.

5.4.6.2 TFS is not correlated with flexion withdrawal reflex activity

Facial behaviour was compared against the incidence and magnitude of flexion withdrawal reflex activity using the TFS components of the PIPP scoring system.

(1) Incidence of flexion withdrawal and visible changes in facial behaviour

Flexion withdrawal reflex activity is not predictive of facial motor activity following a noxious heel lance. Sterile-water treated infants exhibited flexion withdrawal reflex activity in addition to a clear facial response, where TFS>0, following every heel lance (100%; 15/15). However, sucrose-treatment did not reduce the frequency of flexion withdrawal reflex activity (95%; 18/19) despite a lower occurrence of facial activity (58%; 11/19) after a heel lance. The infant who did not exhibit flexion withdrawal reflex activity following a noxious heel lance also failed to mount a change in facial expression over the duration of the 30s recording period.

(2) Latency to facial expression change and reflex response

The latency to peak activity was compared against the time to first observed facial response following a heel lance. Infants who did not exhibit a facial response within the first 5s after the stimulus were excluded from the analysis (sterile-water, n=1/15; sucrose, n=7/18). Correlation analysis showed no significant relationship between these two latency measures for sterile-water (R^2=0.01; p=0.71) and sucrose (R^2=0.03; p=0.58) using Pearson's Correlation.

Of the 7 sucrose infants who did not exhibit a facial response within the first 5s after the noxious stimulus, the mean latency to peak EMG activity was 964.3ms (95% CI 653.3-1275.0ms); the data followed a normal distribution. There were no significant differences when compared against sucrose treated infants with facial response within the first 5s after the stimulus [1042ms (95% CI 755.6-1328.0ms]; unpaired t-test, p=0.70.

(3) TFS and magnitude of reflex activity

There was no relationship between TFS and magnitude of flexion withdrawal reflex activity (Figure 5-14); this was clear in both sucrose and sterile-water treated infants. Correlation analysis confirmed no significant relationship between these variables for sterile-water (R^2=0.17; p=0.13) or sucrose (R^2=0.006; p=0.76) using Pearson's Correlation.

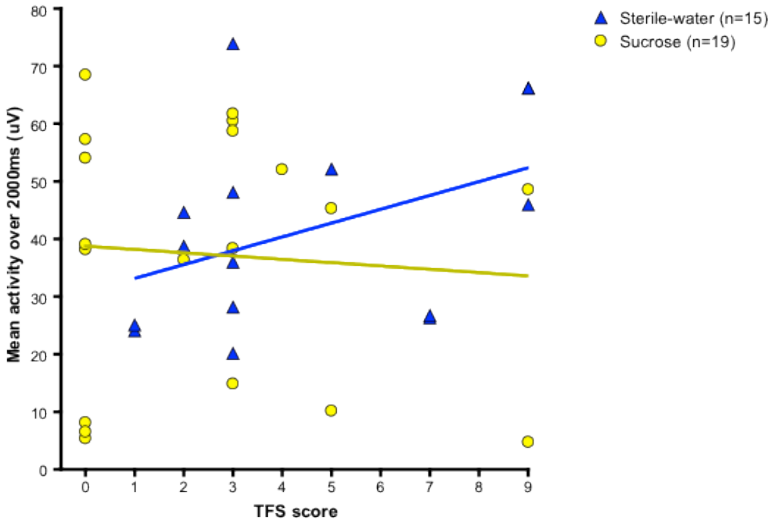


Figure 5-14: No relationship between size of TFS score and flexion withdrawal reflex activity
Note: seven infants (all treated with sucrose) did not score above 0 despite the presence flexion withdrawal reflex activity following a noxious heel lance. Blue triangles represent individual infants treated with sterile-water; yellow circles represent individual infants treated with sucrose.

Summary 4

- Flexion withdrawal reflex activity is evoked in sucrose-treated infants and is independent of the observed facial motor activity. These data support the findings from Study 1 that there is no clear relationship between limb withdrawal and facial expression following a noxious heel lance.
- In infants who do not exhibit a clear facial behaviour, the pattern of activity, magnitude, and latency to reflex response and to peak amplitude were not reduced when compared against infants who exhibit distinct facial motor response to a noxious heel-lance.

5.4.7 Summary of results

(1) A randomised controlled trial was conducted to investigate the effect of oral sucrose administration (0.5ml at 24% sucrose) prior to a noxious heel lance compared to sterile water (control) using surface EMG recordings of flexion withdrawal reflex activity from the biceps femoris. For comparison, clinical pain assessment scores (PIPP) were also recorded.

(2) Oral administration of 0.5ml 24% sucrose at two-minutes prior to a noxious heel lance does not modulate flexion withdrawal reflex activity in full-term infants.

(3) Flexion withdrawal reflex activity characteristics were comparable between treatment groups. Sucrose had no effect on latency properties, pattern of activity or magnitude of flexion withdrawal reflex activity when compared to sterile-water (control).

(4) Sucrose does not attenuate contralateral flexion withdrawal reflex properties in full-term infants. Noxious-evoked flexion withdrawal reflex activity is a bilateral response consisting of synchronous ipsilateral and contralateral biceps femoris activity in sucrose and sterile-water (control) treated infants.

(5) Sucrose administration significantly lowers clinical pain assessment (PIPP) scores. In addition a lower frequency of the evoked facial activity was observed.

(6) Flexion withdrawal reflex activity is evoked in sucrose-treated infants and is not predictive of the degree of observed facial motor activity following a noxious heel lance. There is no clear relationship between limb withdrawal and facial expression following a heel lance, independent of treatment group (these data support the results from Study 1).

5.5 Discussion

In this chapter, a randomised controlled trial was conducted to investigate the effect of oral sucrose administration prior to a noxious heel lance compared to sterile water (control) using direct recordings of flexion withdrawal reflex activity in full-term infants. Distinct from previous randomised-controlled trials examining sucrose, this study simultaneously incorporated quantitative motor reflex activity, facial expression and autonomic responses as outcome measures for the first time in the human infant.

The initial aim of this chapter was to characterise flexion withdrawal reflex EMG activity in sucrose and sterile-water (control) treated infants, and to use this information to determine treatment effectiveness on spinal cord excitability; the present results provide important novel findings that oral sucrose does not modulate flexion withdrawal reflex properties in the full-term infant. These data were supported by parallel activity measured from the contralateral biceps femoris in sucrose and sterile-water treated infants. Secondly, the study investigated the effect of sucrose on clinical pain assessment scores (based on behavioural and physiological measurement); these results concur with the findings from previous randomised controlled clinical trials and demonstrate significantly lower PIPP scores when compared to sterile-water, (Stevens *et al.*, 2010). Finally, the relationship between flexion withdrawal reflex activity, PIPP scoring and facial activity were investigated. These findings showed no clear relationship between the properties of the flexion withdrawal EMG activity, visual observations of facial motor activity and PIPP scoring. Importantly, whilst these results are in agreement with previous work and show that sucrose administration significantly decreases clinical pain scores, these data additionally demonstrate that oral sucrose does not modulate spinally mediated flexion withdrawal reflex activity in the full-term infant following a noxious stimulus.

5.5.1 Effect of sucrose on human infant flexion withdrawal reflexes

Oral sucrose administration two minutes prior to a noxious heel lance reduces clinical pain assessment (PIPP) scores which are a composite measure of the behavioural and physiological responses to pain, and are in agreement with data from previously reported clinical trials (Stevens *et al.*, 2010). Critically, these results provide additional evidence that sucrose has no effect on nociceptive transmission at the spinal cord. An infant treated with oral sucrose is

capable of mounting a leg withdrawal response to noxious stimulation that is comparable in frequency of occurrence, latency and magnitude to an infant treated with sterile-water. These results highlight the importance of interpreting behavioural and physiological measures of infant pain with caution and the need for more objective tools that determine nociceptive-specific activity to investigate the effectiveness of analgesics in the neonate.

The current results show that flexion withdrawal reflex activity is unaffected by sucrose when compared to sterile-water (control) intervention. The flexion withdrawal reflex in the adult rat and human has been shown to be useful as a measure of central nociceptive processing and in pharmacological testing (Willer, 1985; Woolf, 1983; Woolf *et al.*, 1985). Adult reflex activity is associated with the degree of pain sensation and a dose-dependent reduction in activity occurs with analgesics such as morphine (Willer, 1985). In the present trial, flexion withdrawal reflex activity was evoked in >95% of test occassions and agree with results from Study 1 in this thesis to show that neonates are capable of mounting a robust and reproducible reflex response to a noxious stimulus. Although pain perception cannot be determined in the infant, the size of the reflex response and degree of gross body movement is associated with the degree of stimulus intensity (Abdulkader *et al.*, 2008a; Andrews *et al.*, 1999).

In this study, no differences in the flexion withdrawal reflex EMG properties were detected between treatment groups. Furthermore, the present results show that activation of biceps femoris activity is bilateral and this is independent of treatment groups. The lack of effect is not due to a general insensitivity of the immature flexion reflex to pharmacological modulation. Pharmacological studies have demonstrated that modulation of nociceptive transmission at the levels of the spinal cord can occur in young rat pups and human infants. Animal studies have shown that local anaesthetic blockade of the sciatic nerve, using bupivicane, increases sensory threshold to mechanical stimulation (Kohane *et al.*, 1998), and application to the spinal cord alleviate experimentally induced inflammatory pain (Howard *et al.*, 2001). Reflex limb withdrawal to mechanical and thermal stimulation of the hindpaw shows the attenuation of spinal sensory responses in rat pup at P3, P10 and P21 following morphine administration (Nandi *et al.*, 2004). Further, the α-adrenergic agonist, dexmedetonimine, reduced mustard oil induced hyperalgesia of the paw and surrounding skin region at these ages (Walker *et al.*, 2007). Few pharmacological studies have examined the modulation of flexion withdrawal reflex activity in the human infant due to the array of study design and ethical issues accompanied with clinical trials (Anand *et al.*, 2005). The local anaesthetic effect of topical amethocaine gel causes a reduction in cutaneous reflex sensitivity

and can last for up to 5 hours (Jain *et al.*, 2000a; Jain *et al.*, 2000b). Additionally, reversal of heightened sensitivity has been demonstrated with topical administration of the local anaesthetic cream, EMLA, when applied to the region of skin injury (Fitzgerald *et al.*, 1989). Collectively studies examining pharmacological modulation of flexion withdrawal reflex properties in the neonate show that peripheral and central dampening of the neural circuits underlying pain processing can be detected using flexion withdrawal reflex activity.

Animal and human studies demonstrate that the properties of the flexion withdrawal reflex in the neonate are different to that of the adult. Motor coordination of the limb away from the stimulus is much less refined in the neonate. Activity consists of poorly directed movement, in young rat pups this is often toward the site of stimulation (Waldenstrom *et al.*, 2003), and gross, exaggerated movements of all limbs in response to a threshold stimulus has been reported in the kitten (Ekholm, 1967), rat-pup (Holmberg *et al.*, 1996) and human infant (Andrews *et al.*, 2002a; Andrews *et al.*, 2002b; Franck, 1986) [Study 1 of this thesis]. Here we show that noxious heel lance evoked contralateral biceps femoris activity is synonymous with ipsilateral activity, in agreement with a more 'diffuse' and less directed spinal nociceptive response compared to adults. In addition cutaneous sensitivity is greater in the neonate than in the adult and the reflex can be evoked at innocuous levels of intensity, as shown in Studies 1 and 2. Nevertheless the reflex withdrawal evoked by a noxious tissue-damaging stimulus is considerable greater than that produced by touch (Abdulkader *et al.*, 2008a; Andrews *et al.*, 1999; Study 1 of this thesis; Fitzgerald *et al.*, 1988b). However, these developmental differences do not explain the outcome of this study as comparisons were between infants of the same developmental stage and both ipsilateral and contralateral responses were included.

5.5.2 The effect of sucrose on facial expression

Whilst sucrose does not modulate spinal cord excitability, the present results show that it has an inhibitory effect on facial activity. Infants treated with sucrose were less likely to evoke a visible facial response to a noxious heel lance compared to sterile water treated infants. The coordination of facial motor activity is mediated at the brain-stem level by the trigeminal and facial nerve (cranial nerves V and VII). Although the neural circuitry underlying the effects of sucrose is unknown, Anseloni *et al* (2005) found that analgesia elicited by intraoral sucrose in neonatal rat pups is produced by a neural substrate limited to the brainstem and spinal cord. It

is possible that sucrose may mediate inhibition of facial motor activity at the level of the brainstem.

In the adult, spinal flexion withdrawal reflex activity is also influenced by supraspinal systems. Since sucrose is shown to exert calming effects on the infants, perhaps due to distraction but does not affect nociceptive reflexes, it is possible that the influence of the attention/distraction or sleep-state on reflex excitability may develop later in life (see Sandrini *et al.*, 2005 for comprehensive review). Attention to noxious stimulation enhances pain perception whilst distraction decreases reported pain (Arntz *et al.*, 1993; Levine *et al.*, 1982; Miron *et al.*, 1989). In the animal, studies using food as a distraction agent demonstrate that hind-limb withdrawal occurs at longer latencies with less vocalisation in response to brief noxious stimuli during feeding compared to when not feeding (Casey *et al.*, 1983). Pain management strategies in the paediatric units also employ distraction techniques including breast feeding, non-nutritive sucking and maternal contact during acute painful procedures (Johnston *et al.*, 2010), but this is measured by facial expression and not by reflex excitability. During sleep, flexion withdrawal reflex activity is more stable; in humans, flexion withdrawal reflex activity evoked during sleep show an increased latency and duration of activity compared to the wakeful state (Sandrini *et al.*, 2001; Shahani, 1968). Furthermore, sleep-wake states affect cutaneous reflex activity in the newborn and throughout childhood (Vecchierini-Blineau *et al.*, 1982), but this is likely to employ different pathways from attention/distraction.

The calming effect induced by sucrose in rats is blocked by systemic injection of opioid receptor antagonists (Anseloni *et al.*, 2002; Blass *et al.*, 1987). In the adult, the mechanism of opioid action is thought to decrease activity of descending projections and leads to reduce flexion withdrawal reflex activity. One possibility is that in the infant population studied here, descending controls are immature and such an effect is not possible. Animal studies suggest that maturation of descending control occurs quite late in the preadolescent period; connections from the PAG and RVM, located in the brainstem, only exhibit adult-like inhibitory properties from P21 (Hathway *et al.*, 2009; van Praag *et al.*, 1991). RVM control of the nociceptive activity in spinal cord undergoes a developmental shift from facilitation of spinal reflex activity in the postnatal period to tonic inhibition in the preadolescent period (after three weeks of age); (Hathway *et al.*, 2009). Subcutaenous midazolam application, a positive allosteric modulator of the $GABA_A$ receptor, induces a dose-dependent reduction in mechanical and thermal withdrawal thresholds of the hind-limb in young rat pups (P3) accompanied with a sensitised EMG response and has no effect in adult animals. In young

rat-pups descending control has an excitatory influence on the spinal cord this may be why no effect of sucrose on spinal reflexes was observed in this study.

5.5.3 Pain behaviour in the infant

Whereas pain experience and pain behaviour are linked in adults (Puntillo *et al.*, 2004; Willer, 1977), they may not be in infants. Facial expressions are relatively free of learning bias in the neonate and are thought to represent an infant's innate response to pain (McGrath *et al.*, 2006) but there is no real evidence of this. Earlier results from this thesis (Study 1) support the present findings that there is no clear relationship between infant facial expression and flexion withdrawal reflex activity, although the latter is well established in adult studies to parallel pain experience (Willer, 1977; Willer, 1985). Slater *et al* (2008) showed that while changes in cortical haemodynamic activity are well correlated with clinical pain scores, cortical activity could occur without a concurrent behavioural change, which implies that pain assessment based on behavioural tools alone may not accurately reflect the infant pain experience. It is possible therefore that infants treated with oral sucrose may not show a change in facial expression but are processing the noxious stimulus in the same way in the spinal cord (and cortex) as untreated infants.

5.5.4 Clinical implications

Sucrose has been recommended extensively for acute procedural pain relief on the neonatal unit (Anand, 2001; Lefrak *et al.*, 2006; Stevens *et al.*, 2010). The results here show an absence of effect of sucrose on spinal nociceptive withdrawal reflex activity and suggest that sucrose may not affect higher processing involved in pain perception. In Canada alone, 64% of neonatal units have protocols for administering sucrose for procedural pain management (Taddio *et al.*, 2009b). Of concern here is the short-term and long-term effects of sucrose administration, including the long-term effects of repeated administration in infants who undergo many repeated skin-breaking procedures, and the longitudinal developmental effects on the somatosensory circuitry.

Prolonged effects of neonatal tissue injury exist beyond the neonatal period. Infants exposed to pain in early-life exhibit heightened sensitivity that can last into infancy (Abdulkader *et al.*, 2008b; Taddio *et al.*, 1997). Long-term studies on the neurological development show preadolescent children previously admitted to neonatal intensive care exhibit altered somatosensory perception when compared to age-matched controls (Hermann *et al.*, 2006;

Schmelzle-Lubiecki *et al.*, 2007; Walker *et al.*, 2009a), and this is associated with increased haemodynamic activity in the high pain processing centres e.g. somatosensory cortex, insular cortex (Hohmeister *et al.*, 2010). Global changes in thermal and mechanical sensitivities are proposed to be due to centrally-mediated alterations in nociceptive pathways (Walker *et al.*, 2009a). Collectively these data show that injury in early life can change baseline sensory function and enhance responses to future pain.

Of further note, if sucrose was to be administered for every acute painful procedure, the sickest infants, who undergo many clinical procedures, could be exposed to relatively high volumes of sugar during a period of rapid central nervous system development that could equate to up to half a can of Coke per day (Holsti *et al.*, 2010). The use of sucrose over long periods, and relatively high cumulative amount during the entire hospital stay has yet to be evaluated.

5.5.5 Technical considerations

The end-goal of any clinical trial investigating new or currently available approaches to analgesia is the relief of pain, albeit completely or simply a reduction of pain sensation. A key limitation of the present study was the inability to determine actual perception of pain in the neonate. Nevertheless, such a measurement is impossible to verify when investigating clinical effectiveness in a non-verbal population. Clinical pain assessment necessarily depends upon behavioural and physiological measures such as facial expression or cry duration. Although many pain assessment methods are available (Duhn *et al.*, 2004), none of them are considered to be a 'gold standard' as the lack of reliability and objectivity remains problematic (Anand, 2007; Ranger *et al.*, 2007). Moreover, many behavioural and autonomic responses to noxious events result from reflex activity at the spinal cord or brainstem levels and are not a direct indicator of the conscious perception of pain.

The volume/dose of sucrose used in this study was 0.5ml of 24% sucrose and was administered 2 minutes prior to the noxious stimulus. Although precise dosing parameters are not well defined in the literature, those chosen were considered to reflect the optimal dosage that would induce 'analgesia' or certainly lower clinical pain scores. Sucrose volumes ranging from 0.05ml to 0.5ml have been identified as effective in dampening behavioural and clinical pain scores in preterm and full-term infants (Stevens *et al.*, 2010). However, it is the detection of the sweet substance, not the volume, which is required for clinical effectiveness. As a

result, the Cochrane review reports that 0.05ml to 0.5ml is an adequate volume of 24% to 25% sucrose for clinical effectiveness in preterm and full-term infants (Stevens *et al.*, 2010). In human infants, the peak action of oral sucrose administration occurs after 2 minutes, and the duration of action is approximately 5-10 minutes (Blass *et al.*, 1995).

5.5.6 Conclusion

Here, we performed a randomised controlled trial at UCH to investigate the effectiveness of oral sucrose upon nociceptive flexion withdrawal reflex activity and found that it had no effect. Observational measures of pain assessment, such as those using facial expression should be interpreted with caution. Sucrose may increase physiological stability without being directly analgesic.

Chapter 6

General discussion & conclusions

6 General discussion and conclusions

This thesis investigated the development of cutaneous flexion withdrawal reflexes in preterm and full-term human infants using surface EMG recordings of the lower limbs. Detailed discussions of the experimental results are included in Chapters 3, 4 and 5, and are summarised in this concluding chapter.

(1) Characterisation of the flexion withdrawal reflex using surface EMG recordings in preterm and full-term infants

In Chapter 3 (Study 1), the properties of the flexion withdrawal reflex were characterised following noxious and non-noxious mechanical stimulation, with the aim of understanding the selective effects of noxious procedures upon spinal circuits in preterm and full-term infants. The results established that:

- Lower limb flexion withdrawal reflex activity is evoked by a noxious mechanical stimulus to the heel in both preterm and full-term infants. The amplitude of the EMG response and the latency to peak EMG response were significantly larger in preterm infants than full-term infants.
- Flexion withdrawal reflex activity was specific to noxious stimulation in ~70% of babies In a sub-population of preterm and full-term infants (~30%) non-noxious, tactile stimulation of the heel also evoked a significant reflex response.
- The nociceptive flexion withdrawal reflex in infants is a bilateral response, with synchronous ipsilateral and contralateral motor activity evoked in preterm and full-term infants.
- While all infants exhibited flexion withdrawal reflex activity, following a heel lance, only 87% displayed a change in facial expression to the same stimulus.
- There was no clear relationship between the properties of flexion withdrawal reflex EMG activity and observed facial behaviour.

The information obtained for each EMG recording in preterm and full-term infants provided an objective, quantitative method to analyse motor activity. The data from this chapter provided baseline characteristics of flexion withdrawal reflex activity in the neonate for use in subsequent chapters investigating the effect of increasing stimulus intensity, repeated stimulation and modulation of spinal reflex activity with oral sucrose.

(2) Cutaneous sensory thresholds following single and repeated stimulation

The results from Study 1 indicated that the flexion withdrawal reflex could be evoked by low-intensity innocuous stimulation. Study 2 investigated these findings further, whereby a series of graded von Frey hairs were used to determine cutaneous reflex sensitivity in preterm and full-term infants, and simultaneously validate surface EMG recordings of muscle activity with observations of clear limb withdrawal. Furthermore, plasticity of the spinal cord to repeated mechanical stimulation was investigated, with the aim of understanding the effects of repeated handling and cutaneous mechanical disturbance upon spinal circuits in preterm and full-term infants. The results show that:

- Cutaneous sensory threshold, as determined by visible leg withdrawal, rises with increasing gestational age consistent with previous studies (Abdulkader *et al.*, 2008a; Andrews *et al.*, 1999; Andrews *et al.*, 1994; Fitzgerald *et al.*, 1988b).
- The associated flexion withdrawal reflex activity was measured and used as a control to identify changes in reflex activity during a series of repeated stimuli.
- Repeated stimulation at suprathreshold intensities (1/10s for 2 minutes) led to changes in reflex activity that were characterised by the appearance of a greater long lasting after-discharge in preterm infants compared to full-term infants.
- Subsequent sensory threshold testing indicated a developmental change in sensitivity to further stimuli. While full-term infants exhibited a decrease in sensitivity or habituation following repeated stimulation, preterm infants did not habituate but maintained the same level of sensitivity.

The data from this chapter provided thresholds and baseline characteristics of the human infant flexion withdrawal reflex to graded mechanical stimulation. Furthermore it revealed that repeated mechanical stimulation in preterm infants tends to 'wind up' reflex activity and that preterm infants do not display habituation after repeated stimulation as full-term infants do.

(3) The effect of oral sucrose upon spinal flexion withdrawal reflex activity

In Chapter 5 (Study 3), the modulation of cutaneous flexion withdrawal reflex activity by oral sucrose was investigated, in a randomised controlled trial, with the aim of understanding the effects of a commonly administered analgesic upon spinal circuits in preterm and full-term infants. The results show:

- Oral sucrose had no effect on nociceptive transmission at the spinal cord; flexion withdrawal reflex properties were comparable between treatment groups.

- Oral sucrose did not attenuate either the ipsi- or the contralateral flexion withdrawal reflex properties. Noxious-evoked reflex activity consisted of a bilateral response with synchronous ipsilateral and contralateral biceps femoris activity in both treatment groups.
- Oral sucrose reduced clinical pain assessment (PIPP) scores, which are a composite measure of the behavioural and physiological responses to pain. In addition, a lower frequency of evoked facial activity was observed. This is consistent with previous reports (Stevens *et al.*, 2010).

The data in this chapter highlights the differing mechanisms underlying spinal nociceptive reflexes and the behavioural and physiological measures of pain that underlie PIPP scores. Interpreting observed body and facial behaviour together with physiological measures should be undertaken with caution. The results suggest that more quantitative and objective tools of nociceptive activity may be more useful in assessing the effectiveness of analgesics in the neonate.

Conclusions

Ethological Perspective

It is interesting to consider what the data in this thesis means from the point of view of ethology –the understanding of infant behaviour in its natural environment. This investigation has provided information about the development of human responsiveness to stimuli in infancy, focusing on situations that are especially relevant to survival (tactile stimulation and noxious stimulation). A neuroethologist might consider that there are innate physiological mechanisms that permit learning while encouraging the infant to attend closely to very specific stimuli. Thus the greater magnitude of the preterm infant flexion reflex, including a strong bilateral response, could indicate the importance of sensory inputs at the early stage of development and the need for strong spinal reflex behaviour to attract the attention of the mother before the brain is sufficiently well developed to allow a planned, integrated response. The heightened responsiveness to touch could indicate the importance of attending to tactile stimulation from the mother for survival. Repeated exposure to noxious stimuli is not an ethologically normal situation – in a natural environment it would not be consistent with survival. Thus the apparent lack of selectivity to tactile and noxious stimulation allows the same spinal circuit to be used efficiently and effectively and to be shaped and adapted by repeated and tactile stimulation at an early stage of development. The lack of habituation to

repeated stimulation in preterm infants indicates that simple learning in spinal circuits has not yet developed in this age group but has developed by full-term. When a noxious stimulus does occur, if the infant is to survive, a robust flexion reflex will then remove the limb from the potentially damaging stimulus and attract as much help as possible.

Clinical Perspective

In our society, few prematurely born infants live in a natural environment, particularly those that are hospitalised because of prematurity, developmental abnormalities, or illness. In this environment, abnormal tactile stimuli and repeated noxious stimuli are an inevitable consequence of clinical care and there is no physiological advantage to these responses. From this perspective, the flexion reflex circuit becomes a method of investigating the response of the infant nervous system to pain and potentially a method of pain measurement through which to improve analgesic management. Thus the reflex provides a 'readout' of spinal tactile and nociceptive processing and the fact that these are developmentally regulated over the preterm to full-term time period provides important insight into the changing profile of touch and pain sensitivity over this time. The most commonly used analgesic agent for acute procedural pain is oral sucrose but this thesis has shown that oral sucrose has no effect upon flexion reflex responses to a noxious heel lance. It is hoped that a systematic approach to understanding pain and repeated handling will improve pain management in this vulnerable population in the future.

Appendix I: Methods Optimisation

Onset latency detection

Pilot analysis was performed on EMG recordings acquired from a set of full-term infants after a noxious heel-lance stimulus (n=19). The purpose was to clearly define flexion withdrawal reflex activity and optimise methods to quantify EMG activity.

Method

EMG recordings were manually inspected by naked eye and the time of onset recorded. The onset of the flexion withdrawal reflex response was identified by a large change in EMG signal variance following stimulus application. Automatic detection of onset latency utilised the standard deviation of the EMG recording over a 50ms sliding window to indicate change in variance. The onset latency was defined as the point in time where the variance exceeded a set-threshold i.e. 3SD above mean baseline activity. The optimal threshold was chosen on the basis of comparability to the visual inspection onset latency values. The data were subject to onset detection by using a series of voltage thresholds ranging from 1 - 4 SD above mean baseline activity.

In a minority of EMG recordings, a short period of transient spiking at the time of stimulus was present (stimulus artefact). The onset latency was detected from 50ms after the stimulus to adjust for the occurrence of stimulus artefact.

Results

Visual inspection of EMG recordings indicated that all infants exhibited a clear flexion withdrawal reflex response. The mean (±SD) onset latency was 381.6±162.3ms, ranging between 98.0ms and 692.0ms. Automatic onset latency detection using showed the optimal threshold setting was 3SD above mean baseline activity (Figure I- A). At this level of sensitivity, flexion withdrawal reflex activity was detected in all EMG recordings and at values akin to those determined after visual inspection; the mean onset latency was 369.1±190.5ms. No significant differences were found between the threshold at 3SD above mean baseline activity and visual inspection (Student's paired t-test, p=0.54).

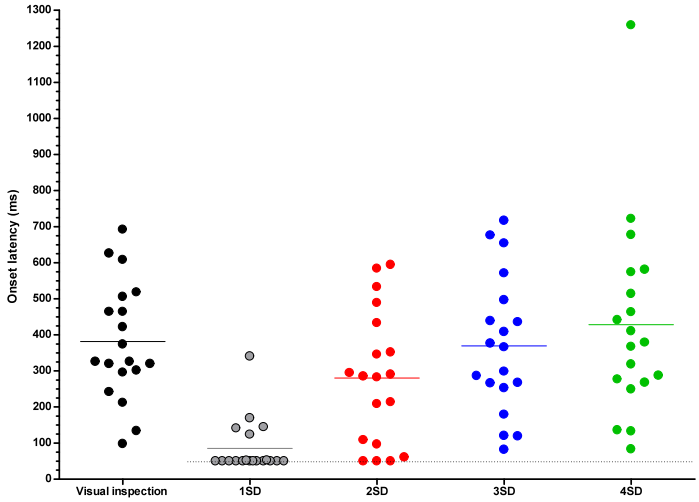


Figure I- A: Onset detection using visual inspection and voltage threshold techniques- the optimal threshold was 3SD above mean baseline

All EMG recordings exhibited clear flexion withdrawal reflex activity when examined by naked eye (black circles). The sensitivity to onset latency decreased as the voltage threshold increased. A low threshold detected transient, small amplitude activity that did not necessarily reflex the onset of a flexion withdrawal reflex response i.e. at 1SD above mean baseline (grey circles), 74% (14/19) exhibited activity that exceeded the threshold at 50ms (minimum time detection limit), these onsets were not detected after visual inspection. A high threshold of 4SD above baseline (green circles) was less sensitive and detected onset of activity at slightly later latencies compared to visual inspection.

Appendix II: Publications

Papers

Slater, R., **Cornelissen, L.***, Fabrizi, L.*, Patten, D., Yoxen, J., Worley, A., Boyd, S., Meek, J. & Fitzgerald, M. (2010). Oral sucrose as an analgesic drug for procedural pain in newborn infants: a randomised controlled trial. *Lancet* **376**(9748)**:** 1225-1232.

Abstracts

Slater, R., **Cornelissen, L.***, Fabrizi, L.*, Patten, D., Yoxen, J., Boyd, S., Meek, J. & Fitzgerald, M. (2010). Oral sucrose as an analgesic drug for procedural pain in newborn infants: a randomised controlled trial. *IASP* (PM220)

Cornelissen, L.L., Slater, R., Boyd, S. & Fitzgerald M (2009). Development of human pain behaviour: Nociceptive flexion withdrawal reflex EMG activity and facial motor responses in preterm and term infants. *Physiology Society (Themed Meeting: Cellular & Integrative Neuroscience)* (PC09)

Cornelissen, L.L., Slater, R., Ingram, R., Boyd, S. & Fitgerald M (2009). Development of human infant pain behaviour: Nociceptive flexion withdrawal reflex EMG activity in preterm and term infants. *Society for Neuroscience* (812.12)

Cantarella, A, Slater, R.L., Patten, D.P., Yoxen, J., Roberts, S**., Cornelissen, L.**, Worley, A., Fitzgerald, M. & Meek, J. (2008). Latency to facial expression change following a noxious procedure is dependent on post-menstrual age in infants from 25 – 43 weeks. *IASP* (PF270)

**these were joint authorships*

Oral sucrose as an analgesic drug for procedural pain in newborn infants: a randomised controlled trial

Rebeccah Slater, Laura Cornelissen, Lorenzo Fabrizi*, Debbie Patten, Jan Yoxen, Alan Worley, Stewart Boyd, Judith Meek†, Maria Fitzgerald†*

Summary

Background **Many infants admitted to hospital undergo repeated invasive procedures. Oral sucrose is frequently given to relieve procedural pain in neonates on the basis of its effect on behavioural and physiological pain scores. We assessed whether sucrose administration reduces pain-specific brain and spinal cord activity after an acute noxious procedure in newborn infants.**

Methods **In this double-blind, randomised controlled trial, 59 newborn infants at University College Hospital (London, UK) were randomly assigned to receive 0·5 mL 24% sucrose solution or 0·5 mL sterile water 2 min before undergoing a clinically required heel lance. Randomisation was by a computer-generated randomisation code, and researchers, clinicians, participants, and parents were masked to the identity of the solutions. The primary outcome was pain-specific brain activity evoked by one time-locked heel lance, recorded with electroencephalography and identified by principal component analysis. Secondary measures were baseline behavioural and physiological measures, observational pain scores (PIPP), and spinal nociceptive reflex withdrawal activity. Data were analysed per protocol. This study is registered, number ISRCTN78390996.**

Findings **29 infants were assigned to receive sucrose and 30 to sterilised water; 20 and 24 infants, respectively, were included in the analysis of the primary outcome measure. Nociceptive brain activity after the noxious heel lance did not differ significantly between infants who received sucrose and those who received sterile water (sucrose: mean 0·10, 95% CI 0·04–0·16; sterile water: mean 0·08, 0·04–0·12; p=0·46). No significant difference was recorded between the sucrose and sterile water groups in the magnitude or latency of the spinal nociceptive reflex withdrawal recorded from the biceps femoris of the stimulated leg. The PIPP score was significantly lower in infants given sucrose than in those given sterile water (mean 5·8, 95% CI 3·7–7·8 *vs* 8·5, 7·3–9·8; p=0·02) and significantly more infants had no change in facial expression after sucrose administration (seven of 20 [35%] *vs* none of 24; p<0·0001).**

Interpretation **Our data suggest that oral sucrose does not significantly affect activity in neonatal brain or spinal cord nociceptive circuits, and therefore might not be an effective analgesic drug. The ability of sucrose to reduce clinical observational scores after noxious events in newborn infants should not be interpreted as pain relief.**

Funding **Medical Research Council.**

Published **Online** September 1, 2010 DOI:10.1016/S0140-6736(10)61303-7

See **Online/Comment** DOI:10.1016/S0140-6736(10)61358-X

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Introduction

International clinical guidelines recommend that oral sucrose is given to relieve procedural pain in neonates.[1] These recommendations are based on results from several randomised controlled clinical trials that conclude that sucrose is effective in reducing pain in preterm and term neonates.[2] Because many infants admitted to hospital undergo repeated invasive procedures,[3,4] and because there is increasing evidence of short-term and long-term adverse neurodevelopmental consequences,[5–9] assessment of the effectiveness of sucrose analgesia in this patient group is essential. As a result, 44 randomised controlled trials investigating the effectiveness of sucrose analgesia in newborn infants are included in a recent Cochrane Review.[2]

A major challenge in analgesic trials in the infant population is definition of a reliable, quantitative outcome measurement of pain because verbal reports and visual analogue scales cannot be used.[5,10] The most commonly used outcome measures are based on behavioural and physiological observations, and many validated composite pain measurement instruments, such as the premature infant pain profile (PIPP), are based on these observations.[11–13] These methods might not, however, be an appropriate outcome measure for neonatal analgesic trials[14–16] because they are largely based on human observation and judgment,[17] and, whereas pain experience and pain behaviour are linked in adults,[18] they might not be linked in neonates. For example, infants who do not display a change in facial expression after tissue damaging procedures might still display significant cortical responses,[19] which suggests that infant behaviour is not a simple indication of pain activity in the brain. Many conditions, such as immaturity, neurological damage, and maternal drug misuse, can affect integrated sensorimotor function and consequent behaviour,[20–22] but might not necessarily affect sensory pain processing.

Single heel lances evoke specific nociceptive brain activity recorded with neonatal electroencephalography (EEG)[9,23] and spinal nociceptive reflexes recorded with electromyography (EMG).[24] We undertook a randomised

controlled trial of sucrose analgesia with use of this specific nociceptive brain activity as a direct measure of infant pain.

Methods

Study design and patients

We undertook a double-blind randomised study on the postnatal ward in the Elizabeth Garrett Anderson and Obstetric Wing, University College Hospital (UCH; London, UK), between Feb 25, 2009, and March 25, 2010. The participants were healthy newborn infants, born at 37–43 postmenstrual weeks, and less than 8 days old.

Medical charts were reviewed and, at the time of study, infants were assessed as clinically stable. All infants were awake; not receiving analgesic drugs, sedatives, or other psychotrophic agents; had not been fed for at least 30 min before start of the study; were supine; and were self-ventilating in air. Infants were not eligible for inclusion in the study if they showed signs of tissue damage on the lower limbs, had previous surgery, had intraventricular haemorrhage or periventricular leukomalacia, were born to diabetic mothers or opioid users, were asphyxiated at birth, or were born with congenital malformations or other genetic disorders. Infants were also excluded if they had contraindications to the administration of sucrose: high risk for necrotising enterocolitis, feeding intolerance, oesophageal atresia or tracheal oesophageal fistula, or active phase persistent pulmonary hypertension.

Ethics approval was obtained from the UCH ethics committee and informed written parental consent was obtained before each procedure. The study conformed to the standards set by the Declaration of Helsinki and Good Clinical Practice guidelines.

Randomisation and masking

Treatment randomisation and dispensing was done offsite at the UCH pharmacy. The pharmacy received the sterile sucrose solution (24% sucrose in purified water) and sterile water samples from Inspiration Healthcare (Respironics, Murrysville, PA, USA) in individual 15 mL vials, which could not be visually distinguished by the packaging. 60 samples were randomised by a block design with a 1:1 allocation for sucrose and sterile water. The samples were separated into six blocks of ten, in which each block contained five sucrose samples and five sterile water samples. The pharmacy labelled each sample with a randomisation code that corresponded to the identity of the solutions. Randomisation was achieved with a computer-generated randomisation code. Only the hospital pharmacy had access to the randomisation codes that could be used to identify the solution. A sealed copy of the randomisation chart was also stored in the neonatal unit in case an adverse event was reported. Throughout the study the researchers, clinicians, participants, and parents were masked to the identity of the solutions. Inspiration Healthcare was not involved in the study design, data collection, or data analysis. No interim analysis was done in this study.

Procedures

The noxious stimulus was a heel lance done to collect a clinically necessary blood sample. The foot was not squeezed for at least 30 s after the heel lance to ensure that the recorded responses could be timed from one discrete event. This procedure was done without impairing the clinical blood collection. No heel lances were done solely for the purpose of the study. Before the administration of the sucrose or sterile water a non-noxious control stimulus was applied to the infant's heel with a heel lancet. The lancet was rotated by 90° and placed against the heel, so that when the spring-loaded blade was released it did not contact the infants' heel. Infants experienced only the non-noxious tactile sensation and auditory click that occurs when the blade is released.

The sucrose solution or sterile water was administered directly onto the anterior surface of the tongue with a 1 mL syringe 2 min before a heel lance was done. In line with present clinical practice, the dose and expiry date of each solution was double-checked by two neonatal practitioners, and a neonatal nurse administered all solutions. A neonatal nurse was present during the studies and was responsible for the clinical care of the infants at all times. She was also responsible for reporting any adverse events to the consultant in charge.

Experimental recording techniques

A neonatal EEG cap (WaveGuard EEG cap, Advanced NeuroTechnology, Enschede, Netherlands) was used to record EEG activity in the infants. 32 recording electrodes were positioned according to the modified international 10/20 electrode placement system at Fz, Fp1, Fp2, F3, F4, F7, F8, FT9, FT10, FC5, FC6, Cz, CPz, C3, C4, CP3, CP4, CP5, CP6, Pz, POz, P3, P4, P9, P10, PP07, PP08, T7, T8, Oz, O1, and O2. Reference and ground electrodes were placed at FCz and the chest, respectively. Electrode to skin impedance was kept to a minimum by massaging the head with an EEG prepping gel before the cap was placed on the head. Conductive EEG gel was placed in the electrode cups, with a syringe, before the EEG cap was placed on the head. The EEG cap was sterilised after each study by the UCH Sterile Services Department.

EEG activity, from 0·05 to 70 Hz, was recorded with the Neuroscan (Scan 4.3) SynAmps2 EEG/EP recording system (Compumedics US, Charlotte, NC, USA). Signals were digitised with a sampling rate of 2 kHz and had a resolution of 24 bit. A 50 Hz notch filter was used. The heel lance was time-locked to the EEG recording with an accelerometer attached to the upper surface of the lancet.

Respiration was monitored with a movement transducer placed on the abdomen and heart rate measured with

www.thelancet.com Published online September 1, 2010 DOI:10.1016/S0140-6736(10)61303-7

lead 1 electrocardiograph (ECG) electrodes placed on the chest. Oxygen saturation and heart rate were continuously measured with a Nellcor N-560 transcutaneous pulse oximeter (Covidien-Nellcor and Puritan Bennett Boulderm, CO, USA) that was placed on the foot contralateral to the site of stimulation. Pulse oximetry data were downloaded from the oximeter to a recording computer as they were acquired.

EMG activity, from 1 to 500 Hz, was recorded from the ipsilateral biceps femoris muscle with self-adhesive bipolar surface silver/silver chloride (Ag/AgCl) electrodes. Electrode to skin impedance was reduced by rubbing the skin with an EEG prepping gel. Electrodes were secured with self-adherent wrap and electrode leads were tied together to minimise electrical interference.

Facial expression was recorded with a portable tripod-mounted camcorder. A light emitting diode (LED), which flashed when either the non-noxious control event or noxious heel lance was done, was placed in the field of view of the camcorder. Figure 1 shows the experimental time line.

Outcome measures

The primary outcome measure was pain-specific brain activity evoked by one time-locked heel lance, recorded with EEG and identified by principal component analysis (PCA). Secondary measures were baseline behavioural and physiological measures, observational pain scores (PIPP), and spinal nociceptive reflex withdrawal activity. The primary hypothesis was that administration of sucrose 2 min before a heel lance would reduce the evoked nociceptive-specific brain activity.

1500 ms EEG epochs corresponding to a noxious heel lance and non-noxious control event were considered for analysis (PCA). The EEG epochs included activity recorded 500 ms before and 1000 ms after each event. The segments were referenced to a common average, baseline corrected, high-pass filtered above 0·5 Hz, and aligned between 400 and 750 ms to correct for interindividual latency variability (maximum –20 to 50 ms). Infants were excluded from the analysis if technical failure occurred in the EEG recording or if movement artifact—defined as a voltage change greater than 50 μV over 50 ms in 15 or more electrodes—was identified in the alignment window.

Nociceptive-specific activity was characterised at electrode site Cz because we have previously reported nociceptive-specific activity at this electrode site,[9,23] and because studies in adults show that similar potentials recorded at Cz are sensitive to pharmacological analgesic drugs.[25]

In the original research protocol we intended to use peak-to-peak amplitude detection. We deviated from the original protocol as instead we used PCA to analyse the data. This is an improved analytical technique that has been successfully used in EEG analysis and is more robust than peak-to-peak amplitude detection because it takes into account the overall signal rather than two single data points that can be easily affected by noise and are difficult to identify. We have successfully used this method in previous work to characterise evoked potentials generated after noxious and non-noxious stimulation in the human infant brain.[9,23]

PCA was used to decompose the EEG epochs recorded at Cz into basic waveforms, termed principal components.[26] Epochs were considered as variables and timepoints as observations; the resultant covariance matrix was selected as the association matrix. The principal components represent systematic variation in the amplitude of the signal across timepoints in a cluster

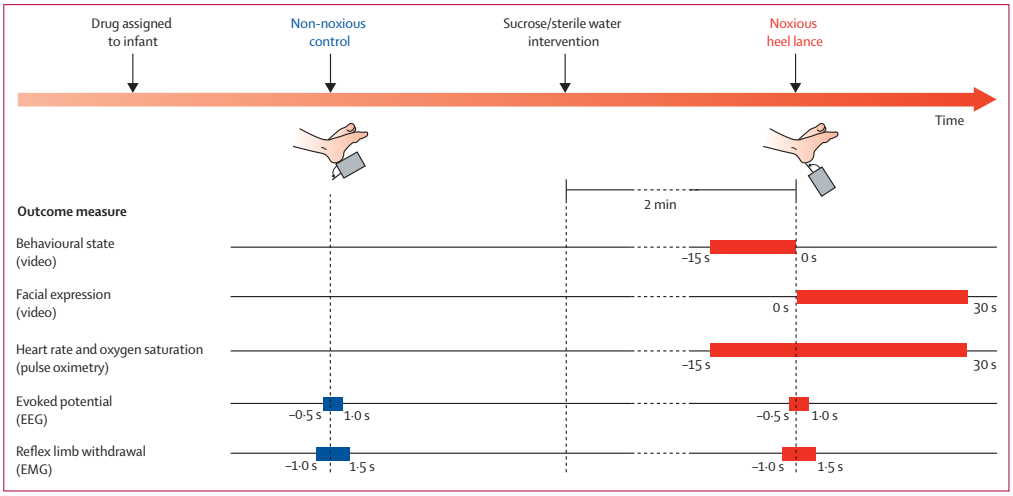

Figure 1: **Experimental time line**
EEG=electroencephalography. EMG=electromyography.

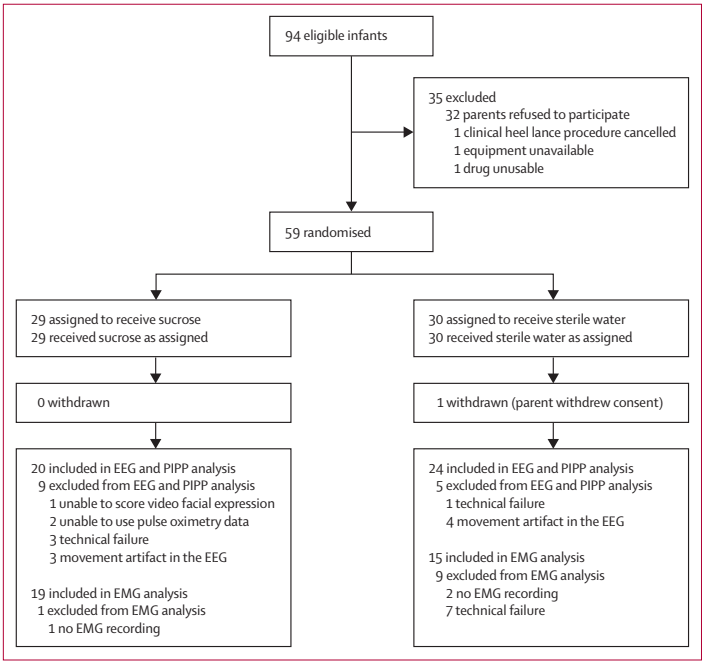

Figure 2: **Trial profile**
EEG=electroencephalography. PIPP=premature infant pain profile. EMG=electromyography.

	Sucrose (N=20)	Sterile water (N=24)
PMA at birth (weeks)	39·8 (1·1)	39·8 (1·3)
PMA at time of study (weeks)	40·1 (1·1)	40·3 (1·4)
Postnatal age at time of study (days)	3 (2)	3 (2)
Birthweight (g)	3449 (453)	3454 (443)
Boys	11/20 (55%)	15/24 (63%)
Apgar score at 1 min	8·2 (1·5)	8·0 (1·9)
Spontaneous vaginal delivery	8/20 (40%)	11/24 (46%)
Right heel lanced	13/20 (65%)	12/24 (50%)

Data are mean (SD) or n/N (%). PMA=postmenstrual age.

Table 1: **Characteristics of participating neonates**

of epochs. The extent to which each component is represented in an individual EEG epoch is quantified by a unique weight. The weights of the first two principal components, which accounted for 84% of the total variance, were considered for analysis.

The PIPP score was calculated for each infant on the basis of behavioural and physiological observations.[12] The facial expression component of the PIPP was analysed by trained observers (two neonatal research nurses) watching the videos for 30 s immediately after the heel lance. The videos were cut into 45 s epochs, which included 15 s before stimulus and 30 s after stimulus. The presence of each facial expression (nasolabial furrow, eye squeeze, and brow bulge) was assessed individually. The number of infants who had a facial expression score of zero was also calculated.[22] The observers were fully masked to the treatment allocation and stimulus type when they analysed the videos. The observers did not know whether they were viewing video footage after the heel lance or the control stimulation. An additional level of masking was imposed by mixing the videos with other non-trial video footage. The facial expression component of the PIPP was rescored by two observers in 15 of 60 (25%) of the videos to assess intra-rater and inter-rater reliability, which was analysed by the Bland-Altman method.[27] Bland-Altman plots showed good reliability with little bias (intra-rater bias 0·07; inter-rater bias 0·73). The limits of agreement for the intra-rater re-test were ±1·62. The limits of agreement for the inter-rater comparison were ±1·49.

The mean heart rate and oxygen saturation in the 15 s before the heel lance, and the maximum heart rate and the minimum oxygen saturation in the 30 s after the heel lance were used to calculate the physiological indices in the PIPP score. Custom-made data analysis software, written in MATLAB, was used to automatically calculate the mean baseline heart rate and oxygen saturation. The software also calculated change in heart rate and oxygen saturation from the heel lance. The data used these parameters to automatically calculate the physiological indices in the PIPP score.

The behavioural state score was calculated by observing the infant's sleep state and facial movements on the video footage in the 15 s before the heel lance. The latency to facial expression change was defined as the latency when the first PIPP facial feature (ie, nasolabial furrow, eye squeeze, and brow bulge) was observed after the heel lance in each infant.[22]

EMG analysis

2500 ms EMG epochs corresponding to a noxious heel lance were considered for analysis. The EMG epochs included activity recorded 1000 ms before and 1500 ms after each event. The segments were high-pass filtered above 10 Hz and rectified. Latency to flexion withdrawal reflex activity was defined as the time after stimulus when the EMG activity exceeded three standard deviations of the pre-stimulus baseline. Magnitude of flexion withdrawal reflex activity was measured in 250 ms periods after stimulus by calculation of the root mean square activity. The summary parameter for the magnitude of the spinal nociceptive reflex EMG activity was defined as the root mean square activity in 1000 ms after stimulus. Infants were not included in the EMG analysis when technical failure occurred in the EMG recording.

Statistical analysis

Data were analysed according to the trial protocol. A sample size of 40 infants had greater than 80% power to

	Sucrose (N=20)	Sterile water (N=24)	p value
Primary outcome			
Nociceptive-specific brain activity (mean weight)	0·10 (0·04–0·16)	0·08 (0·04–0·12)	0·46
Secondary outcomes			
Mean baseline heart rate (bpm)	132·6 (124·3–140·9)	131·8 (122·2–141·5)	0·90
Mean baseline oxygen saturation (%)	99·4% (98·8–100·1)	97·4% (95·0–99·8)	0·13
Baseline behavioural score (from PIPP)	1·3 (0·8–1·7)	1·3 (0·8–1·8)	0·91
PIPP score	5·8 (3·7–7·8)	8·5 (7·3–9·8)	0·02
Latency to change in facial expression (s)	3·8 (1·3–6·4)	3·5 (1·0–6·1)	0·86
Facial non-responders	7/20 (35%)	0/24 (0%)	<0·0001
Mean nociceptive reflex withdrawal activity (μV)	36·11 (24·20–48·02)	30·82 (18·51–43·13)	0·49
Mean latency to nociceptive reflex withdrawal activity (ms)	363·3 (256·4–470·1)	413·5 (262·0–564·9)	0·56

Data are mean (95% CI) or n/N (%). bpm=beats per min. PIPP=premature infant pain profile.

Table 2: **Primary and secondary outcomes**

detect a 30% reduction in the amplitude of the nociceptive-specific brain activity as significant ($p<0·05$; two-tailed). We allowed for a total sample size of 60 infants (30 per group) to account for a high number of anticipated losses (ten per group) because of the possibility that technical failure could occur in any one of the physiological recordings (ie, video, EEG, or pulse oximetry; typically 20%; four per group) and that movement artifact could be recorded in the EEG (typically 20%; four per group).

Two-way nested analysis of variance was applied to the principal component weights to assess the effect of stimulation type (non-noxious control, noxious heel lance) and treatment (sterile water, sucrose). With this analysis we assessed whether noxious stimulation generated a specific component and whether this component was affected by sucrose or sterile water administration. Nociceptive-specific brain activity was identified by measurement of the principal components whose weights were significantly greater after the noxious lance compared with non-noxious control.

As with our previous publications, only one nociceptive-specific principal component was identified. To ensure that the activity defined in our participant sample could be generalised to other external samples, we compared the evoked activity with that reported in a previously published dataset[9] and calculated the correlation coefficient between the observations. Two-way nested analysis of variance was applied in the same way to the root mean square values of the EMG reflex activity.

The effect of the treatment on all other outcome measures was summarised as mean values (with 95% CIs) and significance of the differences tested with unpaired Student's *t* tests. A significance level of 0·05 was used in all the tests. Data were analysed with MATLAB (version 7.8.0).

This study is registered, number ISRCTN78390996.

Role of the funding source

The sponsor of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Figure 2 shows the trial profile. There were no deviations from the inclusion and exclusion criteria. 59 infants were included in our initial sample, of whom 44 were included in the final analysis. Table 1 shows the characteristics of the final population of infants. After sucrose or sterile water administration the mean heart rate, oxygen saturation, and behavioural scores in the 15 s before the heel lance did not differ significantly between infants who were given sucrose and those given sterile water (table 2).

After the heel lance, the PIPP score was significantly lower in infants who were given sucrose than in those given sterile water; however, we recorded no significant difference in the latency to change in facial expression between the two treatment groups (table 2). The number of infants who had a zero facial expression score after the noxious heel lance was significantly higher in the sucrose group than in the sterile water group (table 2).

Nociceptive-specific brain activity was identified by one principal component that was significantly greater after the heel lance (mean principal component weight 0·09 [SE 0·02]) than after the non-noxious control event (0·03 [0·01]; p=0·006; figure 3A). The correlation coefficient between this component and a nociceptive-specific component calculated in an external sample of term infants in a previously published study[9] was 92% ($p<0·0001$). The nociceptive-specific brain activity did not differ significantly between infants who received sucrose (mean principal component weight 0·10 [SE 0·03]) and those who received sterile water (0·08 [0·02]; p=0·46; figure 3B).

Spinal reflex withdrawal activity, recorded by EMG of the biceps femoris of the stimulated leg, was significantly greater after the heel lance (mean activity 33·78 μV [SE 4·02] than after the non-noxious control event

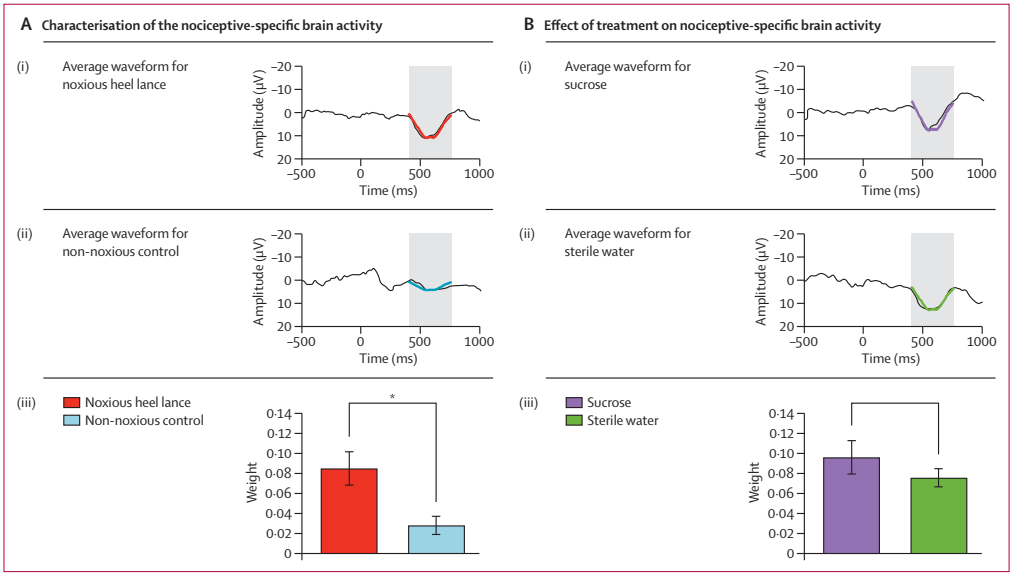

Figure 3: **Characterisation of the nociceptive-specific brain activity (A) and effect of sucrose or sterile water on the nociceptive-specific brain activity (B)**
(A) Average waveform of the group data after (i) noxious heel lance and (ii) non-noxious control stimulus (alignment window 400–750 ms). (iii) Mean (SE) weight of the second principal component after the noxious heel lance and non-noxious control stimulus (*p=0·006). (B) Average waveform of the group data after the noxious heel lance, separated into two groups: (i) infants administered sucrose and (ii) infants administered sterile water (alignment window 400–750 ms). (iii) Mean (SE) weight of the nociceptive-specific component in the sucrose and sterile water groups (p=0·46).

(8·84 μV [3·67]; p<0·0001; figure 4A). The magnitude of the spinal reflex withdrawal activity did not differ significantly between infants who received sucrose (mean activity 36·11 μV [SE 5·67]) and those who received sterile water (30·82 μV [5·74]; p=0·49; figure 4B). Furthermore, mean latency to nociceptive reflex withdrawal activity did not differ significantly between the two groups (table 2). No adverse events were recorded during this study.

Discussion

This randomised controlled trial measured the effect of oral sucrose on procedural pain in infants, with direct measures of brain and spinal cord activity as an outcome measure for pain. The results show that although, as previously reported, sucrose significantly reduces the PIPP score—a composite observational behavioural and physiological measure[2]—it has no effect on the neural activity in sensory pain circuits in the brain or the spinal cord. Although true pain perception cannot be measured in non-verbal populations, neural activity in nociceptive pathways is a more direct measure than behavioural and physiological assessment. The finding that sucrose does not change neural activity strongly suggests that pain perception is not affected by this intervention.

Any measure of pain in this group is necessarily indirect, and whether the electrophysiological measures reported in this study are indicative of the conscious pain experience of the newborn infant cannot be shown. Nevertheless, behavioural and physiological output measures need integration and control of several somatic motor and autonomic circuits, which are affected by several developmental homoeostatic and external factors.[10,19–21] By comparison, the nociceptive-evoked activity in the brain and spinal sensory circuits recorded in this study are a more direct measure of pain activity in the infant CNS. In the adult brain, the magnitude of nociceptive-evoked potentials directly correlates with perceived pain intensity.[28,29] The nociceptive-evoked activity in flexor muscles, which cause reflex withdrawal of the limb, is also a proven functional measure of spinal and supraspinal pain processing.[30] Pharmacological analgesic drugs, such as tramadol and morphine, depress nociceptive-evoked brain activity and flexion reflexes, and result in concurrent reduction in perceived pain intensity in adults.[25,31,32]

Our results accord with previous trials showing that sucrose decreases infant PIPP scores,[2] but they show that such scores do not reliably reflect nociceptive activity in the brain of newborn infants, possibly because of the site of action of sucrose in the brain. The reduction in nociceptive behaviour recorded after oral sucrose in young rats[33] persists after midbrain transection, suggesting that forebrain neural circuits are not needed for this effect.[34] Sucrose reduction of nociceptive withdrawal reflexes in adult rats—a conditioned effect of all hedonistic foods thought to prevent eating from ending—also involves brainstem endogenous inhibitory mechanisms.[35] Thus in infants exposed to noxious procedures, sucrose could mediate a brainstem inhibition

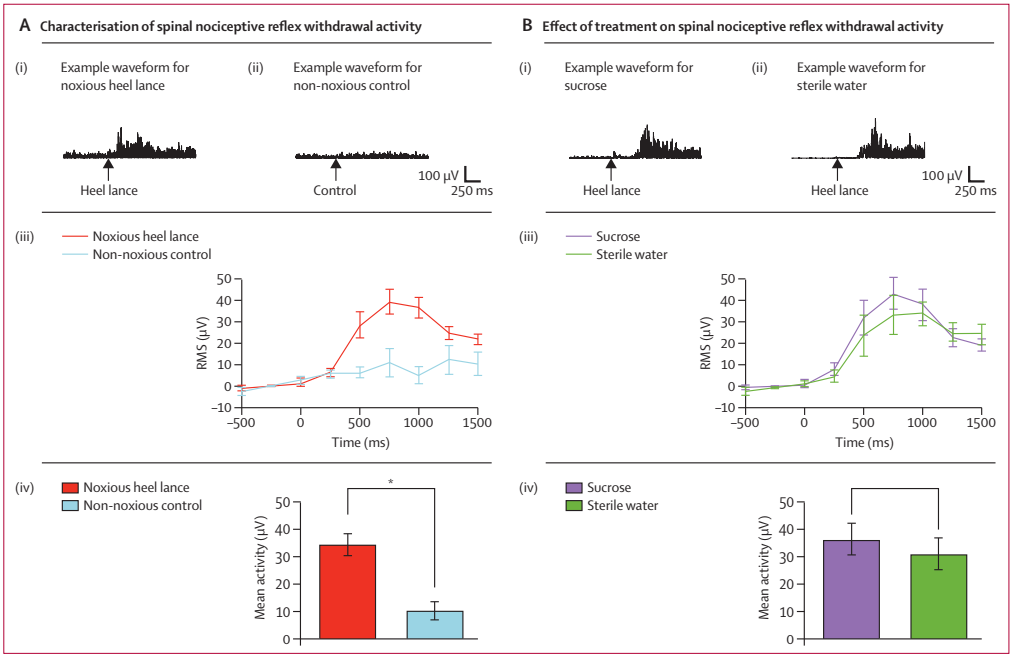

Figure 4: **Characterisation of the spinal nociceptive reflex withdrawal activity (A) and effect of sucrose or sterile water on spinal nociceptive reflex withdrawal activity (B)**

(A) Example spinal reflex withdrawal activity in one infant after: (i) noxious heel lance and (ii) non-noxious control stimulus. (iii) Magnitude (mean [SE]) of the spinal reflex withdrawal activity after noxious heel lance and non-noxious control stimulus represented as the root mean square (RMS) activity in 250 ms time periods in infants. (iv) Mean (SE) spinal reflex withdrawal activity in infants after the noxious heel lance and non-noxious control stimulus (*p<0·0001). (B) Example spinal nociceptive reflex withdrawal activity after a noxious heel lance in two infants who received: (i) sucrose and (ii) sterile water. (iii) Magnitude (mean [SE]) of the spinal nociceptive reflex withdrawal activity after noxious heel lance in infants given sucrose or sterile water represented as the RMS activity in 250 ms time periods. (iv) Mean (SE) spinal nociceptive reflex withdrawal activity in infants given sucrose or sterile water (p=0·49).

of behaviour, and inhibit facial motor activity, while strong pain activation still occurs in the forebrain. This notion is especially important in view of the increasing evidence for short-term and long-term adverse effects of infant pain experience on neurodevelopment.[5–9] The absence of evidence for an analgesic action of sucrose in this study, together with uncertainty over the long-term benefits of repeated sucrose administration,[36] suggest that sucrose should not be used routinely for procedural pain in infants without further investigation.

This double-blind randomised controlled trial used new electrophysiological methods to assess the effectiveness of analgesic drugs in newborn infants. The conclusions that we can draw from this study are limited by the small sample size (n=44), which could mean that this study was not powered to observe subtle effects that sucrose might have on CNS processing. Significant group differences in infant nociceptive brain activity have, however, been recorded in sample sizes of only 15 infants.[9] This single-centre trial should be repeated in a larger sample of infants, and this new method used to test the effect of other known pharmacological analgesic drugs, such as morphine.

In conclusion, our results show that although oral sucrose does reduce observed pain behaviour, it has no significant effect on the magnitude of spinal nociceptive reflexes or on the acute activation of pain networks in the brain. Sucrose seems to blunt facial expression activity after painful procedures, but our data suggest that it does not reduce direct nociceptive activity in central sensory circuits, and therefore might not be an effective analgesic drug.

Contributors

The work presented here was undertaken in collaboration between all authors. RS, AW, JM, SB, and MF defined the research idea and designed the study's method. RS and MF wrote the report. RS, LC, DP, and JY contributed to research data collection and to the study design. DP, JY, and JM were responsible for the clinical care of the participants. RS, LC, LF, DP, JY, AW, SB, JM, and MF reiewed and edited the report. LF, RS, and LC did the statistical analysis. All authors have seen and approved the final version of this report.

Conflicts of interest

We declare that we have no conflicts of interest.

Acknowledgments

This work was supported by the Medical Research Council. We thank Siân Roberts (UCL, London, UK) for help with data acquisition, the UCL/UCH Comprehensive Biomedical Research Centre, the staff at UCH, and all the parents and infants who took part in this study.

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www.thelancet.com **Published online September 1, 2010 DOI:10.1016/S0140-6736(10)61303-7**

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